Serum proteomic analysis revealed diagnostic value of hemoglobin for nonalcoholic fatty liver disease

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Background & Aims: Nonalcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases worldwide. The two linked studies presented herein aimed to identify and verify new biomarkers for NAFLD.

Methods: First, 70 serum samples were analyzed using proteomics approaches to identify potential biomarkers for NAFLD. Second, a total of 6944 initial NAFLD-free subjects were followed up for 3 years to evaluate the predictive value of hemoglobin for NAFLD.

Results: In the first study, 20 differentially expressed protein peaks (11 up-regulated and nine down-regulated) were observed in NAFLD patients upon comparison to the controls. With the aid of bioinformatic tools, we established a biomarker pattern for NAFLD with a sensitivity of 89% and a specificity of 83%. Further analysis suggested a protein peak to be hemoglobin subunit alpha. In the second study, prospective analysis showed that subjects with higher baseline hemoglobin levels were associated with higher incidence of NAFLD. Cox proportional hazards regression analyses showed that the age, gender, and body mass index adjusted hazard ratio (95% CI) for subjects with baseline hemoglobin subunit alpha. In the second study, prospective analysis showed that subjects with higher baseline hemoglobin levels were associated with higher incidence of NAFLD. Cox proportional hazards regression analyses showed that the age, gender, and body mass index adjusted hazard ratio (95% CI) for subjects with baseline hemoglobin level in quintile 2, 3, 4, and 5 vs. quintile 1 was 1.36 (1.02–1.81), 1.66 (1.23–2.25), 1.76 (1.28–2.41), and 1.83 (1.33–2.53), respectively.

Conclusions: Our study showed that serum hemoglobin may have significant predictive value for NAFLD.

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Introduction

Nonalcoholic fatty liver disease (NAFLD), which includes a range of conditions from nonalcoholic steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis, is the most common liver disease identified in Western countries [1,2]. NAFLD has also become a significant form of chronic liver disease in developing countries [3,4]. The development of NAFLD is closely associated with central obesity, type 2 diabetes, hypertension, and dyslipidemia, which form a cluster of metabolic disorders that is now recognized as the metabolic syndrome [5,6]. For this reason, NAFLD has been regarded as a hepatic manifestation of the metabolic syndrome [5].

The diagnosis of NAFLD is suspected in patients with elevated serum aminotransferases and, in many cases, it is suspected in patients with evidence of the metabolic syndrome [7]. Ultrasonography is widely used in clinical diagnosis of NAFLD. However, the sensitivity of ultrasonography is relatively low in patients with mild steatosis. In addition, inter-observer variability is another factor that may limit the usefulness of ultrasonography [8]. Computed tomography and magnetic resonance imaging may help in determining the presence and amount of fatty infiltration in the liver, whereas these techniques cannot be used to accurately determine the severity of liver damage [8,9]. To date, liver biopsy remains the gold standard for NAFLD diagnosis. However, its invasive nature, the observations of significant side effect profile, and susceptibility of this technique to sampling error ultimately raise the need for finding out reliable diagnostic biomarkers to replace liver biopsy [10,11].

Recent advances in the field of proteomics have provided us with powerful tools for studying disease diagnosis and for discovering biomarkers [12]. Among the different technologies available, surface enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF-MS) is an attractive technique since it provides protein profiles in an easy and rapid manner from small amounts of protein, obtained from various biologically complex samples [13]. In addition to this, with the aid of bioinformatics tools, the data gleaned from SELDI-TOF-MS can be further analyzed and successfully used for identification of novel diagnostic biomarkers [14,15]. Recently, Trak-Smyraya et al. [16] firstly applied SELDI-TOF-MS to identify serum markers of steatosis and NASH in extremely obese patients who were candidates for bariatric surgery. However, to date, the
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combination of SELDI-TOF-MS and bioinformatics approach has not been widely applied to the field of biomarker identification for general NAFLD patients.

In this study, we first aimed to identify new biomarkers for NAFLD using a combination of SELDI-TOF-MS and bioinformatics tools, and then verified the predictive value of these potential biomarkers for NAFLD by a population-based study.

Patients and methods

This study was designed to identify and verify new biomarkers for NAFLD in two separate studies. The first study (Study 1) was a proteomic study designed to clarify serum protein fingerprint of NAFLD patients, and identify potential biomarkers for NAFLD. Once the results of Study 1 were obtained, we then sought to determine whether the biomarker identified in Study 1 has a predictor value for NAFLD. For this question, we conducted Study 2, a population-based prospective study among 6944 initially NAFLD-free subjects.

Study 1: Proteomic study

Subjects included for serum proteomic analysis

In order to clarify serum protein fingerprint of NAFLD patients, 70 subjects (35 NAFLD patients and 35 controls) were recruited at the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou Sixth People’s Hospital, Ningbo Yinzhou People’s Hospital, and Ningbo Medical Treatment Center Libu hospital. The diagnosis of NAFLD was based on the following criteria: (i) the hepatic histology indicated the presence of steatosis affecting more than 5% of the total hepatocytes; (ii) the patient had a history of alcohol consumption of less than 140 g/day for men and less than 70 g/day for women; (iii) the patient did not have a specific disease that could lead to hepatic steatosis, such as viral hepatitis, drug-induced liver disease, total parenteral nutrition, autoimmune hepatitis, and metabolic/genetic liver diseases. The subjects included as controls were those with normal anthropometry parameters, blood pressure, liver function tests, liver ultrasound examination, and normal hepatic histological findings. A total of 35 eligible NAFLD patients and 35 healthy controls were enrolled in this study. The mean age and gender of subjects in control group were comparable with those patients from the NAFLD group. Informed consent was obtained from all the subjects and the study protocol was approved by the Hospital Ethics Committee.

Clinical examinations

Clinical examinations were conducted in a standard manner as previously described. Fasted blood samples were obtained from an antecubital vein and the samples were used for the biochemical values analysis and proteomic analysis. The biochemical values were measured using an Olympus AU640 autoanalyzer (Olympus, Kobe, Japan) using standard methods. The biochemical values included alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), fasting plasma glucose (FPG), and serum uric acid (SUA).

Liver biopsy specimens from the subjects were read under coded identification by a liver pathologist who was unaware of the patient details or biopsy sequence. Histological features were interpreted according to Brunt et al. [19], with modification. Steatosis was graded as follows: mild (steatosis involving less than 30% of hepatocytes), moderate (30–60% of hepatocytes affected), severe (more than 60% of hepatocytes affected). Severity of lobular inflammatory, hepatocellular necrosis, portal tract inflammation, pericellular fibrosis, portal fibrosis, and bridging fibrosis were also recorded [19]. NASH was defined as either the presence of steatosis with mixed lobular inflammation and hepatocellular ballooning, as proposed during the AASLD single topic conference [2], or the presence of fibrosis with any stage of fibrosis [20].

Proteomic analysis

The ProteinChip used was the CM10 (weak cation exchange). The detailed protocols for sample preparation were listed in Supplementary subjects and methods. The chips were detected on the ProteinChip Reader (Ciphergen Biosystems). The bioinformatics and biostatistics were described as reported previously [14], and listed in Supplementary subjects and methods. The whole process was performed using the Cancer Institute of Zhejiang University-ProteinChip Data Analysis System (ZUCI-PDAS, www.zlzx.net) designed by Yu Jiekai and based on the MATLAB Web Server. The ProteinChip used was the CM10 (weak cation exchange). The detailed proteomic analysis was performed by 2-DE and MS based methods as described in Supplementary subjects and methods.

Study 2: Prospective study

Subjects and study design

To evaluate the predictive value of hemoglobin for NAFLD, a population-based prospective study was conducted among the employees of Zhenhai Refining & Chemical Company Ltd., Ningbo, China beginning in 2006. The study population was described in detail in our previous uric acid study [21]. A total of 6944 subjects (4496 male and 2448 female) with serum hemoglobin data available in the uric acid study were included in this study. The study protocol was approved by the Hospital Ethics Committee.

Baseline examinations and outcome assessments

Baseline examinations and outcome assessments were performed as previously described [21]. In brief, baseline examinations included a medical history and health habit inventory taken by a physician, anthropometric measurements, hepatic ultrasonic examination, and biochemical measurements. Serum hemoglobin level was analyzed by SYSMEX XT-1800i hematology autoanalyzer according to the manufacturer’s instructions. The diagnosis of fatty liver was based on the results of abdominal ultrasonography using a Toshiba Nemio 20 sonography machine with a 3.5-MHz probe (Toshiba, Tokyo, Japan).

The diagnosis of metabolic syndrome was based on the new International Diabetes Federation definition [22]. For a person to be defined as having the metabolic syndrome they must have: central obesity (defined as waist circumference >90 cm for Chinese men and >80 cm for Chinese women), plus any two of the following four factors: (i) raised triglyceride level, defined as triglycerides >1.7 mmol/L or specific treatment for this lipid abnormality; (ii) reduced HDL-C level, defined as HDL-C <1.03 mmol/L in males and <1.29 mmol/L in females; (iii) raised blood pressure, SBP >130 mm Hg or DBP >85 mm Hg, or treatment of previously diagnosed hypertension; (iv) raised FPG, defined as FPG >5.6 mmol/L, or previously diagnosed type 2 diabetes.

Statistical analysis

Continuous variables are presented as mean and standard deviation (SD) or median and interquartile range (IQR), as appropriate. Continuous variables were compared with Wilcoxon rank-sum test, student’s t-test, Kruskal-Wallis H test or one-way analysis of variance, depending on the normality of the data; categorical variables were compared using the χ² test. The Kolmogorov-Smirnov test was used to assess whether continuous data were normally distributed.

To explore the association between hemoglobin level and risk for incident NAFLD, subjects were stratified according to their baseline hemoglobin levels: quintile 1, <125 g/L; quintile 2, 126–134 g/L; quintile 3, 135–142 g/L; quintile 4, 143–149 g/L; and quintile 5, >150 g/L. The baseline characteristics of the subjects in each quintile were compared. Kaplan-Meier estimator was used to estimate the cumulative incidence of NAFLD.

We used Cox proportional hazards regression analyses to estimate hazard ratios for incident NAFLD for each baseline hemoglobin quintile over the whole follow-up range. The Cox proportional regression model assumes that the effects of the predictor variables are constant over time. This was assessed by tests based on the generalization by Grambsch and Therneau using scaled Schoenfeld residuals [22]. The subjects within the first quintile were used as reference group. The data were first adjusted for age and gender and then for other covariates that might confound the relationship between the hemoglobin and NAFLD. For linear trends of risk, the number of quintiles was used as a continuous variable and tested on each model.

The statistical analyses were performed using the SPSS software package version 13.0 for Windows (SPSS Inc., Chicago, IL). p <0.05 (2-tailed) was considered to be statistically significant.

Results

Study 1

Clinical and laboratory data

Results from analysis of the NAFLD patients and controls indicated that they were different in terms of BMI, waist
circumference, ALT, AST, triglyceride, total cholesterol, LDL cholesterol, FPG, and SUA, while there was no difference in terms of age, gender, type 2 diabetes, systolic blood pressure, diastolic blood pressure, or HDL cholesterol between the two groups (Table 1).

Among 35 NAFLD patients, mild steatosis, moderate steatosis, and severe steatosis was observed in 23, 7, and 5 patients, respectively. Among the 12 patients with moderate and severe steatosis, NASH was observed in three patients. No abnormal hepatic histological finding was observed in control subjects. No abnormal hepatic histological findings were illustrated in Supplementary Fig. 1.

Serum biomarker identification for NAFLD
In order to clarify serum protein fingerprint of NAFLD patients, 70 serum samples from 35 histologically confirmed NAFLD patients and 35 controls were analyzed by ProteinChip based SELDI-TOF-MS. We first evaluated the reproducibility of the ProteinChip by using eight serum samples from one single subject, which were subsequently applied to each chip in a random fashion. The coefficient of variance for selected peaks after being normalized subsequently applied to each chip in a random fashion. The coefficient of variance for the selected peaks after being normalized was 0.05%.

By intensity was 14.5% and the coefficient of variance for selected peaks after being normalized was 0.05/507 = 0.0000986, \( p \) value. The comparison of proteins profiles obtained from NAFLD patients and controls showed a total of 20 differentially expressed proteins peaks. Eleven of these peaks were highly expressed in NAFLD patients whereas nine of these peaks were low expressed.

The top 10 differentially expressed protein peaks were selected, randomly combined, and input into the support vector machine. Among these, the peaks with \( m/z \) of 2760, 2957, 2967, 3269, 3403, 5814, and 6306 Da were significantly low expressed in NAFLD patients, while the peaks with \( m/z \) of 8567, 13,756, and 15,124 Da were significantly high expressed in NAFLD patients (Supplementary Fig. 2). The accuracy of all models was calculated and the diagnostic model that achieved the highest Youden’s index was selected as the final model to distinguish the two groups. The diagnostic model to differentiate NAFLD patients from controls was comprised of six biomarkers with \( m/z \) of 2760, 2957, 2967, 5814, 6306, and 15,124 Da, which was supported to be hemoglobin subunit alpha, was significantly up-regulated in NAFLD group (Supplementary Methods and Results); while the other five peaks were significantly down-regulated. The diagnostic model had a sensitivity of 89% and a specificity of 83% as evaluated by leave-one cross-validation.

Study 2
Baseline characteristics
At baseline, 6944 subjects (4496 male and 2448 female) were included, whereas 466 subjects (317 male and 149 female) did not complete the 3-year follow-up examination. All subjects did not have competing events including death during the 3-year follow-up. Baseline characteristics were not significantly different between the subjects lost to follow-up and those with successful follow-up (Supplementary Table 1). The baseline characteristics of subjects in each hemoglobin quintile are shown in Table 2. Age, male gender, BMI, waist circumference, blood pressure, serum liver enzymes, serum lipids, FPG, and SUA all tended to increase at higher hemoglobin levels (all \( p <0.001 \)). In contrast, HDL-C decreased with as hemoglobin increased (\( p <0.001 \)).

Association of hemoglobin with incident NAFLD
To explore the association of hemoglobin with incident NAFLD, subjects were stratified into quartiles according to their baseline

<table>
<thead>
<tr>
<th>Variable (years)</th>
<th>Controls</th>
<th>NAFLD patients</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>48.3 (9.6)</td>
<td>49.1 (13.8)</td>
<td>0.772</td>
</tr>
<tr>
<td>Gender (male/female, n)</td>
<td>14/21</td>
<td>19/16</td>
<td>0.338*</td>
</tr>
<tr>
<td>Type 2 diabetes (yes/no, n)</td>
<td>5/30</td>
<td>6/29</td>
<td>0.108*</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22.24 (2.44)</td>
<td>26.16 (3.21)</td>
<td>0.002</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>78.0 (5.5)</td>
<td>93.7 (9.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>121.0 (2.7)</td>
<td>129.3 (14.4)</td>
<td>0.343</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>78.7 (9.2)</td>
<td>81.7 (10.1)</td>
<td>0.642</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>21.5 (15.5-44.0)</td>
<td>57.0 (21.0-104.0)</td>
<td>0.003*</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>22.5 (18.3-27.8)</td>
<td>32.0 (22.0-50.0)</td>
<td>0.011*</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>4.28 (1.85-4.67)</td>
<td>4.97 (2.97-3.27)</td>
<td>0.001*</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.22 (0.78)</td>
<td>5.01 (1.37)</td>
<td>0.004</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.09 (0.90-1.34)</td>
<td>1.18 (1.04-1.39)</td>
<td>0.237*</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.20 (1.85-2.67)</td>
<td>2.97 (2.48-3.27)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/L)</td>
<td>4.86 (4.51-5.70)</td>
<td>5.39 (4.95-6.08)</td>
<td>0.044*</td>
</tr>
<tr>
<td>Serum uric acid (mmol/L)</td>
<td>277.7 (92.3)</td>
<td>350.6 (75.7)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Data are expressed as mean (SD) or median (IQR). *\( \chi^2 \) test; †Wilcoxon rank-sum test; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
hemoglobin levels. The cumulative incidence of NAFLD was calculated by dividing the number of cases by the numbers of subjects in each hemoglobin quintile.

A total of 773 subjects developed NAFLD during the follow-up study. Kaplan–Meier estimator showed that the cumulative incidence of NAFLD increased progressively with increased baseline hemoglobin level (Fig. 1). This observation indicated that the subjects with higher baseline hemoglobin levels were more likely to develop NAFLD than those with lower levels.

**High hemoglobin predicts increase risk of NAFLD**

To estimate hazard ratios of each hemoglobin quartile for incident NAFLD, Cox proportional hazards regression analyses were applied. As shown in Table 3, with comparison to the subjects in quintile 1, baseline hemoglobin levels were positively correlated with the hazard ratios for incident NAFLD. After adjustment for age and gender, with comparison to subjects in quintile 1, the hazard ratios (95% CI) for subjects in quintile 2, quintile 3, quintile 4, and quintile 5 were 1.43 (1.08–1.90), 1.88 (1.39–2.55), 2.21 (1.61–3.03), and 2.42 (1.76–3.34), respectively (p for trend <0.001; Table 3). The relationship of hemoglobin with incident NAFLD remained significant even when further adjusted for five components of metabolic syndrome, which are all major cofactors that impact the development of NAFLD (Table 3).

When mean hemoglobin increases, the prevalence of anemia should decrease accordingly. In the second study, at total of 212 subjects fulfilled the diagnostic criteria of anemia (Hb <120 g/L for male and Hb <110 g/L for female) at baseline evaluation. With comparison to non-anemic subjects, the 3-year cumulative incidence of NAFLD was significantly lower for anemic subjects (4.2% vs. 11.36%; p <0.001). In age and gender adjusted Cox analysis, anemia was showed to be a protective factor that reduced the development of NAFLD, the hazard ratios (95% CI) was 0.50 (0.26–0.96). These results indirectly indicated that hemoglobin level is an important factor that predicts the development of NAFLD and the risk increases with increase in baseline hemoglobin levels.

**Discussion**

In this study, we first analyzed serum samples using SELDI-TOF-MS and bioinformatics tools, and successfully established a serum protein fingerprint diagnostic model for NAFLD. A further population-based prospective study showed that serum hemoglobin level independently predicts the development of NAFLD and may serve as a biomarker for the disease.

The field of biomarker is an area of fast growing interest in the setting of NAFLD [23]. As it has been well established, the serum contains thousands of proteins or peptides that regulate a large number of physiologic functions [24]. The alteration of specific proteins or peptides may be related to a specific disease [25]. Therefore, the identification of the protein patterns in the serum could provide a valid clinical diagnosis of many diseases. Traditional “knowledge-based” approaches such as enzyme-linked immunosorbent assay based assays are classical and reliable methods to achieve biomarker identification [26]. However, these

### Table 2. Baseline characteristics of study subjects according to hemoglobin quintiles.

<table>
<thead>
<tr>
<th>Baseline hemoglobin quintile</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n = 1454)</td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>0.001</td>
</tr>
<tr>
<td>Gender (male/female, n)</td>
<td></td>
</tr>
<tr>
<td>Type 2 diabetes (yes/no, n)</td>
<td></td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td></td>
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<tr>
<td>Waist circumference (cm)</td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
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<tr>
<td>Alanine aminotransferase (U/L)</td>
<td></td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td></td>
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<tr>
<td>Triglyceride (mmol/L)</td>
<td></td>
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<tr>
<td>Total cholesterol (mmol/L)</td>
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<td>HDL cholesterol (mmol/L)</td>
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<tr>
<td>LDL cholesterol (mmol/L)</td>
<td></td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/L)</td>
<td></td>
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<tr>
<td>Serum uric acid (mmol/L)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean (SD) or median (IQR). The subjects were grouped according to baseline hemoglobin quintiles: quintile 1, <120 g/L; quintile 2, 120–125 g/L; quintile 3, 126–135 g/L; quintile 4, 136–142 g/L; quintile 5, 143–149 g/L; and quintile 6, >150 g/L. HDL, high-density lipoprotein; LDL, low-density lipoprotein.

*P* values are computed from *χ²* test for categorical data and on Kruskal–Wallis *H* test or one-way analysis of variance for continuous data, depending on the normality of the data.
The subjects were stratified into quartiles according to their baseline hemoglobin levels; subjects with higher hemoglobin levels had an increased cumulative incidence rate of NAFLD. p value for trend is computed from Cox analysis, the number of quintiles was used as a continuous variable and tested on each model.

Fig. 1. Kaplan–Meier curves reflecting cumulative incidence rate of NAFLD according to quintiles of baseline hemoglobin level. Subjects were stratified into quintiles according to their baseline hemoglobin levels; subjects with higher hemoglobin levels had an increased cumulative incidence rate of NAFLD. p value for trend is computed from Cox analysis, the number of quintiles was used as a continuous variable and tested on the model.

Table 3. Risk of development of NAFLD according to baseline hemoglobin quintiles in unadjusted and adjusted models.

<table>
<thead>
<tr>
<th>Models</th>
<th>Baseline hemoglobin quintile</th>
<th>Unadjusted</th>
<th>Adjusted for age and gender</th>
<th>Adjusted for age, gender, and BMI</th>
<th>Adjusted for age, gender, and T2DM</th>
<th>Adjusted for age, gender, and metabolic diseases(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio (95% confidence interval)</td>
<td>(\chi^2) test</td>
<td>p*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td></td>
<td>1 (n = 1454)</td>
<td>1.54 (1.17-2.02)</td>
<td>2.11 (1.63-2.73)</td>
<td>2.53 (1.97-3.25)</td>
<td>2.76 (2.15-3.55)</td>
</tr>
<tr>
<td>Adjusted for age and gender</td>
<td></td>
<td>2 (n = 1417)</td>
<td>1.43 (1.08-1.90)</td>
<td>1.88 (1.39-2.55)</td>
<td>2.21 (1.61-3.03)</td>
<td>2.42 (1.76-3.34)</td>
</tr>
<tr>
<td>Adjusted for age, gender, and BMI</td>
<td></td>
<td>3 (n = 1384)</td>
<td>1.36 (1.02-1.81)</td>
<td>1.66 (1.23-2.25)</td>
<td>1.76 (1.28-2.41)</td>
<td>1.83 (1.33-2.53)</td>
</tr>
<tr>
<td>Adjusted for age, gender, and T2DM</td>
<td></td>
<td>4 (n = 1376)</td>
<td>1.43 (1.08-1.90)</td>
<td>1.88 (1.39-2.55)</td>
<td>2.20 (1.60-3.02)</td>
<td>2.42 (1.76-3.33)</td>
</tr>
<tr>
<td>Adjusted for age, gender, and metabolic diseases(^a)</td>
<td></td>
<td>5 (n = 1313)</td>
<td>1.37 (1.03-1.82)</td>
<td>1.70 (1.25-2.30)</td>
<td>1.79 (1.30-2.46)</td>
<td>1.74 (1.26-2.41)</td>
</tr>
</tbody>
</table>

The subjects were grouped according to baseline hemoglobin quintiles: quintile 1, ≤125 g/L; quintile 2, 126–134 g/L; quintile 3, 135–143 g/L; quintile 4, 144–149 g/L; and quintile 5, ≥150 g/L. BMI, body mass index; MS, metabolic syndrome; T2DM, type 2 diabetes.

*p values for trend are computed from Cox analysis, the number of quintiles was used as a continuous variable and tested on each model.

*Metabolic diseases, including central obesity, raised triglyceride level, reduced HDL-C level, raised blood pressure and raised FPG, were defined according to the definition recommended by the new International Diabetes Federation definition.
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by a high intake of cereals and vegetables and a low intake of animal foods. This may partially explain why the prevalence of NAFLD is significantly lower in China than in Western countries. Future research should thus examine the association between hemoglobin, serum ferritin level, iron intake, and NAFLD. In addition, due to ethnic differences in dietary habit, the homogeneous ethnicity of the sample should also be born in mind for future related studies [32].

The relationship between serum hemoglobin and NAFLD may be partially modulated by haptoglobin level. As we know, haptoglobin is an acute-phase protein and its secretion increases in response to injury or inflammatory insults. Importantly, haptoglobin acts mainly as an antioxidant, binding to free hemoglobin and thereby inhibiting the hemoglobin-induced oxidative damage to tissues. Because both inflammation and oxidative injury are thought to contribute to the development of NAFLD, haptoglobin has the potential to be a determinant of NAFLD risk and can modulate the risk linked to free hemoglobin levels [33].

A limitation of this study is that serum insulin levels have not been analyzed. Nevertheless, we adjusted for the metabolic syndrome, instead of insulin resistance, in the Cox analysis. As we know, insulin resistance has been repeatedly observed to be significantly associated with metabolic syndrome in many large sample size studies [34–36]. The second limitation is that, due to sample limitation, we could not establish a diagnostic model to distinguish simple steatosis from NASH. Yet despite this, the diagnosis of simple steatosis does have significant clinic importance. By obtaining an early diagnosis for hepatic steatosis, we can start intervention at the very beginning of the disease, thereby decreasing the negative effects caused by the disease. In addition, we can also monitor the effects of different therapeutic interventions in a simple manner that is independent of a liver biopsy. The third limitation is that several important confounders, including urban residence, income, occupation, high serum ferritin, iron intake, energy, protein, and fat have not been examined in this study; further studies are needed to clarify this point. Moreover, further studies are also needed to investigate the diagnostic value of the five remaining biomarkers for NAFLD.

In summary, our results showed that the serum protein fingerprint analysis using the SELDI-TOF-MS technique combined with bioinformatic approaches can help to discover novel biomarkers for NAFLD. Hemoglobin may have a significant predictive value for NAFLD. Further studies on the biomarkers identified herein may eventually help establish a reliable non-invasive tool for diagnosis of NAFLD.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2011.05.027.

References


