Validating candidate biomarkers for different stages of non-alcoholic fatty liver disease

Reem Al-Qarni, MSca,∗, Muhammad Iqbal, PhDb, Maram Al-Otaibi, PhDa, Faisal Al-Saif, MD, MHAa, Assim A. Alfadda, MDb, Hisham Alkaladi, PhDa, Fahad Barmehriz, MDc, Mazen Hassanain, PhDa,d,f

Abstract
Non-alcoholic fatty liver disease (NAFLD) is a common chronic condition caused by the accumulation of fat in the liver. NAFLD may range from simple steatosis to advanced cirrhosis, and affects more than 1 billion people around the world. To date, there has been no effective treatment for NAFLD. In this study, we evaluated the expression of 4 candidate NAFLD biomarkers to assess their possible applicability in the classification and treatment of the disease.

Twenty-six obese subjects, who underwent bariatric surgery, were recruited and their liver biopsies obtained. Expression of 4 candidate biomarker genes, PNPLA3, COL1A1, PPP1R3B, and KLF6 were evaluated at gene and protein levels by RT-qPCR and enzyme-linked immunosorbent assay (ELISA), respectively.

A significant increase in the levels of COL1A1 protein (P = .03) and PNPLA3 protein (P = .03) were observed in patients with fibrosis-stage NAFLD compared to that in patients with steatosis-stage NAFLD. However, no significant differences were found in abundance of PPP1R3B and KLF6 proteins or at the gene level for any of the candidate.

This is the first study, to our knowledge, to report on the expression levels of candidate biomarker genes for NAFLD in the Saudi population. Although PNPLA3 and PPP1R3B had been previously suggested as biomarkers for steatosis and KLF6 as a possible marker for the fibrosis stage of NAFLD, our results did not support these findings. However, other studies that had linked PNPLA3 to fibrosis in advanced NAFLD supported our current finding of high PNPLA3 protein in patients with fibrosis. Additionally, our results support COL1A1 protein as a potential biomarker for the fibrosis stage of NAFLD, and indicate its use in the screening of patients with NAFLD. Further studies are required to validate the use of COL1A1 as a biomarker for advanced NAFLD in a larger cohort.

Abbreviations: COL1A1 = collagen type 1 α 1, KLF6 = Kruppel-Like Factor 6, NAFLD = non-alcoholic fatty liver disease, PNPLA3 = patatin-like phospholipase domain-containing protein 3, PPP1R3B = protein phosphatase 1, regulatory subunit 3 B.

Keywords: biomarkers, fatty liver, fibrosis, non-alcoholic fatty liver disease, obesity, steatosis

1. Introduction
Non-alcoholic fatty liver disease (NAFLD) is characterized by the presence of hepatic steatosis (HS), as determined by histology or imaging, in the absence of fat accumulation factors such as alcohol consumption, Wilson disease, starvation, or parenteral nutrition (PN), according to the American Academy for the Study of Liver Disease (AASLD, 2018). NAFLD is a growing epidemic that occurs along with obesity. It affects more than 20% of the U.S. population, 10% of that in Africa, 29% in South Asia, and 31% in the Middle East,[2,3] affecting more than 1 billion people around the world.[4] Advanced age and male gender have been reported to increase the risk of developing NAFLD.[5] NAFLD can proceed from steatosis to cirrhosis, end-stage-liver-disease, or hepatocellular carcinoma (HCC).[5,6,7] To date, there is no effective treatment for the advanced stages of NAFLD,[6,8] and the annual cost for the care of these patients is estimated to be approximately $103 million in the U.S., £5.24 billion in the U.K., and €27.7 billion in Italy, Germany, and France put together.[10] Therefore, there is an urgent need for improved understanding on the pathogenesis, progression, and outcome of NAFLD, as well as for the development of effective and economical therapeutic interventions.[11,12]

Multiple factors, including genetic polymorphisms, gender, age, ethnicity, and immunity contribute to the progression of NAFLD.[13] Several genes, including Patatin-like phospholipase domain-containing protein 3 (PNPLA3),[14] phosphatase 1, regulatory subunit 3 B (PPP1R3B),[15] collagen type 1 α 1
(COL1A1),[16] and Kruppel-Like Factor 6 (KLF6)[17] have been associated with the development of NAFLD. Previous studies had shown a positive association between the rs738409 variant of PNPLA3 and degree of steatosis in NAFLD.[15,18] Additionally, environmental stress (or unhealthy lifestyle), a major risk factor for the development of NAFLD, has been reported to modulate the expression of specific variants of PNPLA3.[19] Similarly, changes in the expression of PPP1R3B, and that of specific variants, have been correlated to high levels of serum lipids and development of HS.[13,20,21] Furthermore, expression of COL1A1 has been associated with the development of hepatic fibrosis and prognosis[22,23] while KLF6, a member of the Kruppel-Like family of transcriptional factors,[17] has been linked to the activation of hepatic satellite cells (HSCs) following liver injury.[24–26]

Although several genes have been associated with the development and prognosis of NAFLD, they have not yet been developed as biomarkers for the fast and efficient diagnosis of NAFLD, with liver biopsies still considered the gold standard for its diagnosis.[5] Furthermore, expression of gene polymorphisms are known to be population-dependent, and therefore, the expression of candidate biomarker genes should be validated in specific populations, before adopting them for clinical use. In this study, we investigated the expression of PNPLA3 and PPP1R3B in the steatosis stage, and COL1A1, and KLF6 in the fibrosis stage among the NAFLD patients admitted to our hospital, to validate the feasibility of developing these candidate biomarker genes as NAFLD stage-specific biomarkers.

Further, we conducted correlation studies to identify non-invasive biomarkers for NAFLD staging; and assessed relationships between expression biomarkers and clinical or blood parameters for patients with NAFLD, in both steatosis and fibrosis stages. However, staging remains complicated due to the poor understanding of NAFLD pathophysiology. The current study sought to elucidate the genetic diversity associated with NAFLD phenotypes in an effort to improve the current staging methods for this disease.

2. Materials and methods

2.1. Case selection and collection of samples

Twenty-six adult patients with NAFLD, who were severely obese (BMI > 29.9 kg/m²) and underwent bariatric surgery in the hospital between 2012 and 2018, were recruited for the study. All clinical and laboratory data of the patients were used for the study. All liver samples in this study were collected from the liver disease biobank of Liver Disease Research Center (LDRC), which is a multi-site (local) registry with collaborators from multiple countries.

Liver biopsy was performed concurrently with bariatric surgery. Liver tissues were obtained at the start of the surgery to avoid any impact of surgical stress. Thereafter, tissues were embedded in optimal cutting temperature (OCT) compound in cryovials, snap-frozen in liquid nitrogen, and stored at −80 °C in the LDRC biobank. Furthermore, NAFLD classification for the participants was determined based on the findings from radiology and laboratory investigations. These investigations included blood tests (complete blood count (CBC), lipid profile, liver enzymes, and blood sugar level) and X-ray.

Participants’ age ranged from 19 to 60 years, including 11 males and 15 females. The inclusion and exclusion criteria were based on the criteria of AASLD, and the European Association for the Study of Liver Disease for the diagnosis of NAFLD,[27,28] as presented in Table 1. All study participants provided signed informed consent, and the study was approved by the Institutional Review Board (IRB), King Saud University College of Medicine (KSU-MC) (Approval No. E-17–2654). Sample- and data-sharing agreements were signed between the study participants and the Liver Disease Research Center (LDRC), KSU-MC. The LDRC Biobank adhered to the IRB guidelines, the university SOPs for biobanking, and with the Canadian Repository Network.

In this study, we targeted only 2 stages of NAFLD, namely (i) steatosis and (ii) fibrosis.

Therefore, participants with steatosis and fibrosis were screened and randomly selected from the classified liver samples as Normal, Steatosis, and Fibrosis. Control subjects were morbidly obese (BMI ≥ 35), with negative results in the liver biopsy for NAFLD. The control group was similar to the other groups (patients with NAFLD) that underwent liver biopsy during bariatric surgery to diagnose NAFLD.

Approximately 400 patients/samples were screened by LDRC, and 26 NAFLD subjects (15 females and 11 males) were included in this study. Patients were selected according to the inclusion

### Table 1

| Inclusion criteria | Exclusion criteria |
|--------------------|-------------------|
| Age between 18 and 60 years | Age less than 18 and more than 60 years |
| Patients presenting with steatosis and fibrosis stages of NAFLD | Consumption of alcohol |
| Participants classified by the pathologist into NAFLD stages based on the analysis of their liver samples | Patients presenting with the following diseases/medical conditions: |
| | Alcoholic fatty liver disease, Hypothyroidism, Hypopituitarism |
| | Drug-induced liver disease |
| | Other liver diseases (Hepatitis A, B, C, and AIDS) |
| | Starvation |
| | Parenteral nutrition |
| | Celiac disease |
| | Wilson disease |
| | Other causes of fat/triglyceride accumulation, such as hypercholesterolemia |

* Subjects in the inflammatory stage were excluded due to the strong interaction between the steatosis and fibrosis stages, which would make it a challenge on metabolic level to differentiate between the stages.

* Patients were classified into non-alcoholic fatty liver disease based on liver sample analysis as this is currently considered the gold standard for non-alcoholic fatty liver disease diagnosis.

NAFLD = non-alcoholic fatty liver disease
criteria based on previously classified liver samples (Table 1). Inclusion criteria considered adult patients between 19 to 60 years of age, having NAFLD in the absence of any other liver disease. From patients with NAFLD, we selected subjects who were in the steatosis and fibrosis stages of NAFLD; these liver samples were classified by a pathologist.

A total of 26 patients were included in the study, of which 15 samples were used for RT-qPCR, and all 26 were used for enzyme-linked immunosorbent assay (ELISA); 9 obese subjects (No NAFLD Control group), 9 obese subjects with steatosis (steatosis group), and 8 obese subjects with fibrosis (fibrosis group) were enrolled. All 3 groups were age-, sex-, and BMI-matched.

2.2. Real-Time quantitative PCR (RT-qPCR)

Two genes corresponding to steatosis, PNPLA3[15] and PPP1R3B,[29] 2 genes corresponding to fibrosis, COL1A1[30] and KLF6[31] and 1 reference gene (hydroxymethylbilane synthase – HMBS) were selected[32] for expression analysis. Gene sequences were obtained using the RNAseq tool on the NCBI webpage (ncbi.nlm.nih.gov). Primer 3 program was used to design the primers for each gene (Table 2). PCR primers were tested using the in-silico PCR tool provided by the University of California Santa Cruz (UCSC) (https://genome.ucsc.edu/cgi-bin/hgPcr), and optimized in our lab.

Total RNA was isolated from liver tissues using the RNeasy Mini kit (cat. 74104; Qiagen, Hilden, Germany) following the manufacturer’s protocol. DNA contamination was eliminated by using RNase-free DNase (Qiagen). RNA quality and concentration were determined using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Waltham, MA) by measuring the absorbance at 260 and 280 nm. Complementary DNA (cDNA) was synthesized using a high-capacity cDNA reverse transcription kit without RNase inhibitor (cat. # 4368814; Applied Biosystems, Waltham, MA), following the manufacturer’s instructions. The cDNA was stored at −80 °C. Quantitative real-time expression of genes was performed using a 7500 Real-Time PCR System (Applied Biosystems, Singapore) and the Power SYBR Green PCR master mix (cat.# 4367659; ThermoFisher Scientific, Singapore) by measuring the fluorescence at 590–570 nm. The 2−ΔΔCt equation was used to calculate the relative mRNA expression levels.

2.3. ELISA

Total proteins were extracted from liver tissues using standard procedures. Briefly, the liver tissues were rinsed in ice-cold PBS (0.01 mol/L, pH 7.0–7.2). The tissues (approximately 30 mg) were homogenized in microtubes (2 mL) with RIPA buffer (cat. # 89900; Thermofisher Scientific, Waltham, MA) containing Thermo Fisher Scientific protease and phosphatases inhibitor cocktails (cat. # A32961; ThermoFisher Scientific, Waltham, MA) and metal beads using a Qiagen TissueLyser LT (cat. # 85600; Qiagen, Hilden, Germany). The tissue homogenates were then centrifuged for 15 minutes at 5,000 × g in a refrigerated centrifuge. The supernatants were collected, aliquoted, and stored at −80 °C. Total protein concentration was estimated using the Pierce™ BCA Protein Assay kit (cat. # 23225; Thermofisher Scientific, Waltham, MA) following the manufacturer’s instructions. The expression of PNPLA3, COL1A1, PPP1R3B, and KLF6 in the tissue lysates were estimated using ELISA kits from Aviva system biology (Aviva system biology cat. no. OKDC01974; Aviva system biology cat. no. OKEH00542; San Diego, CA) and from MyBioSource companies respectively (mybiosource cat. # MBS9324146, and MyBioSource cat. # MBS9342148; San Diego, CA), following the manufacturer’s protocols.

2.4. Statistical analysis

Data were analyzed using Statistical Package for Social Studies (SPSS 22; IBM Corp., NY). Continuous variables are presented as mean ± standard deviation, and categorical variables are expressed as percentages. Moderate t test and one-way ANOVA were used for continuous variables with a normal distribution. Mann–Whitney U test or Kruskal–Wallis test was used for non-parametric data. Pearson’s correlation coefficient or Spearman’s rank correlation was used to determine the relationship between the variables. Shapiro–Wilk test was used to assess the normality of the data. A P-value < .05 was considered statistically significant.

3. Results

3.1. Demographic and clinical data

Table 3 lists the clinical and demographic characteristics of all the study participants.

| Table 2 | PCR primers and amplicon size. |
|---|---|
| Gene | Gene type | PCR primers (5′ → 3′) | Product size |
| HMBS | Reference gene | Forward: ACCCACAGTGTGAGGATCTC Reverse: AAACCAAGTTATAGGCGATG | 194 bp |
| PNPLA3 | Candidate gene for steatosis stage | Forward: GAAGGATGATGACTCTGAGGTTG Reverse: GGTCCGAGATGCTCTGATG | 197 bp |
| PPP1R3B | Candidate gene for steatosis stage | Forward: TTCTGCAAGGCGTGATG | 216 bp |
| COL1A1 | Candidate gene for fibrosis stage | Forward: CTCTGGAAGGCTGCTGCTG | 166 bp |
| KLF6 | Candidate gene for fibrosis stage | Forward: GGCCTGATGCTGACTAAT Reverse: AGTTGGTCTGCTGTCAGT | 170 bp |

bp = base pair, COL1A1 = collagen type 1 α 1, HMBS = hydroxymethylbilane synthase, KLF6 = Kruppel-Like Factor 6, PNPLA3 = patatin-like phospholipase domain-containing protein 3, PPP1R3B = Protein phosphatase 1, regulatory subunit 3 B.
The mean age of the study participants was 30 years old; their mean BMI was 44.3 kg/m² (morbid/severe obesity), and their mean waist circumference was 126.5 cm (large). In the control group, the age ranged from 19 to 60 years. Control group mean waist circumference was 126.5 cm (large). Patients with the fibrosis stage of NAFLD showed elevated expression of PNPLA3 (Fig. 2A) and COL1A1 (Fig. 2C). There was no significant difference in the expression of either PNPLA3 (Fig. 1A) and PPP1R3B (Fig. 1B) or COL1A1 (Fig. 1C) and KLF6 (Fig. 1D).

Patients with the fibrosis stage of NAFLD showed elevated expression of PNPLA3 (Fig. 2A) and COL1A1 (Fig. 2C) proteins compared to those with the steatosis stage (P < 0.04). There was no significant difference in the expression of PPP1R3B protein in either stages of NAFLD compared to that in the control group (Fig. 2B). In addition, there was no significant difference in the expression of KLF6 protein between the fibrosis stage of NAFLD and the control group (Fig. 2D).

### Parameters assessed for the study population.

| Parameter                        | Control group | Mean     | SD       | Steatosis group | Mean     | SD       | Fibrosis group | Mean     | SD       | P value |
|----------------------------------|---------------|----------|----------|-----------------|----------|----------|---------------|----------|----------|---------|
| Weight (kg)                      |               | 118.71   | 23.82    | 124.77          | 16.69    | 12.06    | 22.40         | .854     |          |         |
| Height (cm)                      |               | 162.22   | 4.94     | 169.64          | 10.36    | 164.63   | 10.18         | .250     |          |         |
| BMI (kg/m²)                      |               | 44.94    | 8.77     | 43.19           | 1.83     | 45.37    | 9.32          | .842     |          |         |
| Waist circumference (cm)         |               | 124.86   | 22.66    | 137.00          | 7.07     | 117.67   | 24.48         | .580     |          |         |
| Waist/Hip ratio                  |               | 0.84     | 0.06     | 1.01            | 0.00     | 0.77     | 0.19          | .285     |          |         |
| ALT (U/L)                        |               | 32.56    | 4.59     | 73.38           | 35.64    | 56.25    | 47.16         | .004*    |          |         |
| AST (U/L)                        |               | 14.67    | 3.71     | 40.63           | 43.97    | 28.25    | 22.64         | .021*    |          |         |
| GGT (U/L)                        |               | 24.00    | 4.78     | 39.00           | 15.94    | 44.17    | 31.66         | .038*    |          |         |
| GGT UNL                          |               | 25.25    | 15.51    | 72.14           | 16.04    | 70.00    | 16.43         | .122     |          |         |
| Albumin (g/L)                    |               | 36.22    | 2.95     | 51.63           | 21.92    | 37.13    | 5.74          | .042*    |          |         |
| Total WBC (per mcL)              |               | 12.96    | 1.51     | 12.13           | 3.88     | 14.00    | 2.09          | .381     |          |         |
| WBC, neutrophils (×10^9/L)       |               | 7.87     | 1.51     | 8.49            | 3.47     | 10.41    | 3.05          | .195     |          |         |
| Platelet count (×10^3/μL)        |               | 3.94     | 1.74     | 6.10            | 3.36     | 6.36     | 2.73          | .248     |          |         |
| Mean corpuscular volume (fL)     |               | 79.11    | 6.10     | 80.10           | 9.79     | 84.39    | 4.95          | .345     |          |         |
| HbA1c (%)                        |               | 5.95     | 1.50     | 5.90            | 0.42     | 6.16     | 1.43          | .942     |          |         |
| Fasting glucose (mmol/L)         |               | 4.60     | 0.41     | 4.30            | 0.14     | 5.75     | 1.20          | .098     |          |         |
| Cholesterol (mmol/dL)            |               | 4.40     | 0.77     | 5.12            | 0.91     | 5.60     | 0.81          | .181     |          |         |
| Triglyceride (mmol/dL)           |               | 0.88     | 0.32     | 1.20            | 0.53     | 2.47     | 1.81          | .028*    |          |         |
| LDL (mmol/dL)                    |               | 2.89     | 0.74     | 3.42            | 0.97     | 3.04     | 0.61          | .450     |          |         |
| HDL (mmol/dL)                    |               | 1.20     | 0.39     | 1.15            | 0.27     | 1.06     | 0.46          | .750     |          |         |

* P value < 0.05.

** Liver enzymes (ALT, AST, GGT), albumin, and triglyceride levels were significant between all subjects.

### 3.2. Analysis of expression of candidate biomarker genes in steatosis and fibrosis stages of NAFLD.

The expression of 4 candidate biomarker genes, 2 each for the steatosis stage (PNPLA3 and PPP1R3B) and fibrosis stage (COL1A1 and KLF6), were evaluated using RT-qPCR. There was no significant difference in the expression of either PNPLA3 (Fig. 1A) and PPP1R3B (Fig. 1B) or COL1A1 (Fig. 1C) and KLF6 (Fig. 1D).

Patients with the fibrosis stage of NAFLD showed elevated expression of PNPLA3 (Fig. 2A) and COL1A1 (Figure 2C) proteins compared to those with the steatosis stage (P < 0.04). There was no significant difference in the expression of PPP1R3B protein in either stages of NAFLD compared to that in the control group (Fig. 2B). In addition, there was no significant difference in the expression of KLF6 protein between the fibrosis stage of NAFLD and the control group (Fig. 2D).

### 3.3. Correlation analysis of selected genes and assessed parameters.

In steatosis stage of NAFLD, PNPLA3 had a positive association with GGT and triglycerides, whereas PPP1R3B had a positive association with BMI. In fibrosis stage, there was a negative relationship of waist circumference with COL1A1 and KLF6. Additionally, there were positive associations between the following parameters:

(i) COL1A1 and LDL;
(ii) KLF6 and creatinine; and
(iii) KLF6 and urea (Table 4).

In our study, we observed a significant relationship between the protein expression of PNPLA3 (P = 0.03) and COL1A1 in the fibrosis stage (P = 0.03), in obese patients with NAFLD. In the control group, there was a positive correlation between PNPLA3 protein expression and levels of hemoglobin (r = 0.78, P = 0.02), MCV (r = 0.72, P = 0.04), and platelets (r = 0.79, P = 0.04). However, there was a negative correlation between the expression of COL1A1 protein and hemoglobin (r = −0.76,
In patients with fibrosis, we observed a positive correlation of \( \text{PNPLA3} \) protein with waist circumference \((r = 0.813, P = .05)\), and with potassium levels \((r = 0.917, P = .004)\), and there was a negative correlation of \( \text{COL1A1} \) protein expression with albumin \((r = -0.749, P = .03)\) levels.

**4. Discussion**

The aim of this study was to validate the use of specific candidate biomarkers for the 2 stages of NAFLD, namely, (i) steatosis and (ii) fibrosis, in liver samples obtained from patients with NAFLD. Expression of \( \text{PNPLA3} \), \( \text{PPP1R3B} \), \( \text{COL1A1} \), and \( \text{KLF6} \), both at gene level and at protein level, were evaluated in patient-derived liver samples. To the best of our knowledge, this is the first study to investigate the expression of these biomarkers, both at gene and protein levels. Our results suggest that \( \text{COL1A1} \) protein may serve as a potential biomarker for differentiation between the fibrosis (advanced) stage and steatosis stage of NAFLD. A previous study had reported \( \text{PNPLA3} \) protein to be related to advanced NAFLD or fibrosis; which explain the significant expression of \( \text{PNPLA3} \) protein in patients with fibrosis.\(^{[33]}\) However, in our study, there was no significant difference in the expression of \( \text{PNPLA3} \), \( \text{PPP1R3B} \), \( \text{COL1A1} \), or \( \text{KLF6} \) in NAFLD.

NAFLD is a growing epidemic that affects approximately 1 billion individuals worldwide.\(^{[34]}\) Liver biopsy is the gold standard method for its diagnosis.\(^{[27]}\) Since 2002, gene expression profiling has been used as a robust diagnostic tool in many diseases, including various cancers.\(^{[35,36]}\) NAFLD has 4 stages; the first of which is steatosis, which involves accumulation of fat in the liver with or without inflammation.\(^{[37]}\) This stage is often reversible by following a healthy lifestyle that promotes weight loss.\(^{[38,39]}\) The second stage is steatohepatitis (SH), which involves the accumulation of fat along with inflammation with or without fibrosis.\(^{[40]}\) Fibrosis is the third stage and the only stage that is unaffected by age or diabetes mellitus (DM).\(^{[41]}\) The fourth stage is cirrhosis, which occurs in 3.5% of patients with NAFLD.\(^{[40]}\) One out of 5 patients with NAFLD proceed from cirrhosis to HCC.\(^{[41]}\)

Steatosis is the only stage of NAFLD that may occur in the absence of other stages.\(^{[42]}\) While steatosis is a clinically stable stage, it generally occurs with some degree of inflammation.\(^{[43]}\) Simple steatosis is an un-progressive condition, and accounts for the first stage of NAFLD.\(^{[44]}\) HS can be defined as the...
accumulation of lipid droplets in more than 5% of hepatocytes.\cite{45} There are 3 types of HS (or fatty liver) –

(i) microvesicular,
(ii) macrovesicular, and
(iii) mixed steatosis.\cite{8}

Mixed steatosis is the most common in patients with NAFLD, in which both micro- and macro-vesicular steatosis are present.\cite{37}

The fibrosis stage is considered a progressive stage in NAFLD.\cite{4} Hepatic fibrosis is a result of 2 types of insults to hepatocytes – cell injury and necro-inflammation.\cite{46} In obese animal models (with insulin resistance), leptin induces the generation of extracellular matrix (ECM) proteins in HSCs, which contributes to fibrosis of the liver.\cite{47,48} Additionally, insulin resistance induces the synthesis of connective tissue growth factor (CTGF), overexpression of which has a fibrogenic effect that is positively related to the degree of fibrosis.\cite{48}

According to the National Health Service (NHS),\cite{49} there is currently no effective treatment for NAFLD.\cite{50} Several randomized clinical trials (RCTs) on different therapeutic interventions for NAFLD are currently at different phases of completion. One

---

**Table 4**

| Stage       | Investigated biomarker | Related parameter | Correlation (r²) |
|-------------|------------------------|-------------------|-----------------|
| Steatosis   | PPP1R3B                | Body mass index (BMI) | 0.81 |
|             | PNPLA3                 | Gamma-glutamyl transferase (GGT) | 0.88 |
|             | COL1A1                 | Triglycerides | 0.96 |
|             | KLF6                   | Waist circumference | -0.83 |
|             | COL1A1                 | - | -0.86 |
|             | KLF6                   | Low-density lipoproteins (LDL) | 0.77 |
|             |                       | Creatinine | 0.83 |
|             |                       | Urea | 0.54 (P= .055, positive trend) |
| Fibrosis    | PNPLA3                 | Potassium | 0.85 |

\(COL1A1 = \) collagen type 1 \(\alpha1\), \(KLF6 = \) Kruppel-Like Factor 6, \(PNPLA3 = \) patatin-like phospholipase domain-containing protein 3, \(PPP1R3B = \) protein phosphatase 1, regulatory subunit 3 B.
of the influential theories in NAFLD is that its treatment is dependent on the disease stage, and that the same medication may not be suitable for all patients with NAFLD. Further, gene expression studies contribute to the identification of novel biomarkers for the diagnosis and treatment of various diseases, including NAFLD. The PNPLA3 gene has been implicated in the progression of NAFLD, with high expression of COL1A1, which has been previously shown in the case of tendons. Therefore, association of high LDL with COL1A1 in the fibrosis stage of NAFLD may serve as a novel discovery. Furthermore, high expression of KLF6 may be associated with impaired or abnormal function. Abnormal renal profile might increase the risk of fibrosis, since the expression of KLF6 gene, which has been implicated in renal disease, increases with high levels of urea and creatinine.

According to our observations in this study, we suggest the following as non-invasive parameters for screening and staging NAFLD:

1. Potential biomarkers for early intervention in NAFLD: in terms of protein expression, we suspect high levels of hemoglobin, platelets, and MCV to possibly increase or indicate a high risk for steatosis or fatty liver. Since CBC could affect the expression of PNPLA3 protein, these blood parameters are potent for NAFLD screening in obese population.

2. Potential parameters for steatosis: high BMI (which affects PPP1R3B), and high TG levels (which affects PNPLA3).

3. Potential biomarkers for fibrosis: waist circumference has an inverse association with fibrosis (by lowering the expression of COL1A1 and KLF6). High LDL affects the expression of COL1A1. In regard to renal impairment, there are 3 suggested parameters including:

   (i) creatinine;
   (ii) urea (which both affect KLF6), and
   (iii) potassium (which related to PNPLA3).

High expression of COL1A1 protein was associated with albuminemia while high expression of PNPLA3 gene and its protein were both related to high potassium levels. This might confirm high potassium levels as a non-invasive biomarker for advanced NAFLD or fibrosis stage. Waist circumference has a negative association with PNPLA3 protein.

According to the data mentioned previously, we assumed CBC to be a potential biomarker for steatosis or NAFLD risk. Such biomarkers should be investigated in a larger population to examine their applicability in early intervention of NAFLD. If suitable, CBC can be used as a screening tool for NAFLD in obese population.

Certain limitations have been noted in this study. For instance, the sample size was comparatively small due to the inadequate number of patients who met the inclusion criteria. Further, most liver biopsies were carried out in 2013, which could have affected the determination of stage and progression of NAFLD. One of the challenges in the study was the use of liver tissues stored in the OCT compound. The OCT solution must be removed from the liver tissue before processing for RNA extraction. We, therefore, recommend the use of tissues frozen by liquid nitrogen as an
alternative to protect RNA from degradation and for more efficient extraction.

NAFLD staging is encouraged in the clinical care of NAFLD, especially with the new pharmacological agents. We believe the classification of patients with NAFLD, based on their gene signatures, would enable the administration of more personalized treatments, and thus enhance outcome of the treatment. However, until 2018, AASLD did not advocate the use of genetic testing in clinical care setting. The American Gastroenterology Association (AGA) also did not advise the screening of patients with NAFLD, in high-risk groups, in the primary care setting and obesity and diabetes clinics. The same recommendation regarding the avoidance of screening goes for the NAFLD screening in families, as per the NAFLD guidelines of the AGA. However, the AASLD has recommended the classification of NAFLD as a topic for future research.

Our results suggest the progression of NAFLD in the liver to be accurately determined based on the expression of a small group of genes and histopathology. Further studies on the expression of these biomarkers would bridge the gaps in the understanding of NAFLD pathogenesis. Gene and protein expression studies might also help in the early diagnosis of NAFLD, and may lead to the discovery of new genetic biomarkers to manage and treat NAFLD. Comparing the expression of genes and proteins in patient serum and tissues, and associating them with the stage of the disease would shed new light on the pathogenesis of NAFLD. Based on our results, we recommend the use of genomic and proteomic evaluations for the staging of NAFLD in RCTs. Most recent RCTs for NAFLD treatment do not support the use of classification systems in NAFLD staging. We hypothesized that genetic and proteomic investigations would aid the development of new guidelines for clinical care of patients with NAFLD. Further studies, using a larger cohort, should be performed to validate the biomarkers identified in this study. Additionally, the relationship between expression of the biomarkers and liver enzymes should be further explored.

Taken together, our results suggest a potential use of COL1A1 protein expression as a classification biomarker for the fibrosis stage of NAFLD, which could help in identifying patients with fibrosis without a liver biopsy. This would help in developing screening and preventive measures for NAFLD in future. However, the prognostic potential of COL1A1 in the fibrosis stage of NAFLD would need further validation in a larger cohort.

5. Conclusion

This study showed the potential use of protein biomarkers for the classification of NAFLD. We anticipate that COL1A1 protein levels could be used to determine disease progression in NAFLD, and differentiate patients with steatosis from those with more advanced fibrosis. ALT and AST levels were significantly associated with the steatosis stage, and mean albumin and TG levels were higher in steatosis subjects. We observed several significant differences in the assessed parameters, especially in the steatosis group. These differences should be further explored to identify new biomarkers for the early diagnosis of NAFLD.

Acknowledgments

We would like to thank Dr. Khalid Al-Regaiey for his constructive suggestions and technical support. We are also grateful to the members of the Stem Cell Unit lab (Department of Anatomy) and Physiology laboratory, Department of Physiology, College of Medicine, king Saud University, for their support and guidance. We would like to thank all members in the Molecular Genetic Pathology lab, in particular Mrs Khouloud Al-Bahool, and Microbiology laboratory, king Saud University-Medical City (KSU-MC). Also, a special thank you for Prince Naif Bin Abdulaziz Health Research Center (King Saud University) and Mr Anhar Ullah (department of Cardiac Sciences, King Fahad Cardiac Center, College of Medicine, King Saud University) for statistical support.

Author contributions

Conceptualization: Reem Al-Qarni, Muhammad Iqbal, Mazen Hassanain.
Funding acquisition and investigations: Reem Al-Qarni.
Methodology: Reem Al-Qarni, Muhammad Iqbal, Mazen Hassanain, and Maram Al-Otaibi.
Project Administration: Reem Al-Qarni, Maram Al-Otaibi, Faisal Al-Saif and Mazen Hassanain.
Resources: Reem Al-Qarni, Muhammad Iqbal, Maram Al-Otaibi, Faisal Al-Saif, Assim A Alfadda, Hisham Alkhalidi, Fahad Bamehriz, and Mazen Hassanain.
Supervision: Reem Al-Qarni and Mazen Hassanain.
Validation: Reem Al-Qarni, Muhammad Iqbal, Maram Al-Otaibi, Assim A Alfadda.
Visualization: Reem Al-Qarni, Muhammad Iqbal, and Mazen Hassanain.
Writing – original draft: Reem Al-Qarni.
Writing – review & editing: Muhammad Iqbal, Maram Al-Otaibi, Assim A Alfadda, Mazen Hassanain.

References

[1] Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the study of liver diseases, and American College of Gastroenterology. Gastroenterology 2012;142:1592–609.
[2] Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Aliment Pharmacol Ther 2011;34:274–85.
[3] Younossi ZM, Loomba R, Anstee QM, et al. Diagnostic modalities for nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, and associated fibrosis. Hepatology 2018;68:349–60.
[4] Albhasi S, Sanyal A. Recent advances in understanding and managing non-alcoholic fatty liver disease. F1000Res 2018;7:F1000 Faculty Rev-720.
[5] Williams CD, Stengel J, Asike MI, et al. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. Gastroenterology 2011;140:124–31.
[6] Younossi ZM, Koenig AB, Abdelatif D, et al. Global epidemiology of nonalcoholic fatty liver disease—meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology 2016;64:73–84.
[7] Dyson JK, Anstee QM, McPherson S. Non-alcoholic fatty liver disease: a practical approach to diagnosis and staging. Frontline Gastroenterol 2014;5:211–8.
[8] Burt AD, Lackner C, Tiniakos DG. Diagnosis and assessment of NAFLD: definitions and histopathological classification. Semin Liver Dis 2015;35:207–20.
[9] Beloso C, Souto J, Fabregat M, et al. Association of TCF7L2 mutation and atypical diabetes in a Uruguayan population. World J Diabetes 2018;9:157–64.
[10] Younossi ZM, Blissett D, Blissett R, et al. The economic and clinical burden of nonalcoholic fatty liver disease in the United States and Europe. Hepatology 2016;64:1577–86.
[11] Kahali B, Halligan B, Speliotes EK. Insights from genome-wide association analyses of nonalcoholic fatty liver disease. Semin Liver Dis 2011;31:175–81.

[12] Younossi Z, Anstee QM, Marietti M, et al. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. Nat Rev Gastroenterol Hepatol 2018;15:11–20.

[13] Berton LC, Adams LA. The natural course of non-alcoholic fatty liver disease. Int J Mol Sci 2016;17:774.

[14] Yuan X, Waterworth D, Perry JRB, et al. Population-based genome-wide association studies reveal six loci influencing plasma levels of liver enzymes. Am J Hum Genet 2008;83:520–8.

[15] Hernandez R, McLean J, Lazo M, et al. Association between variants in or near PNPLA3, GCKR, and PPP1R3B with ultrasound-detected steatosis based on data from the third National Health and Nutrition Examination Survey. Clin Gastroenterol Hepatol 2013;11:1183–90e2.

[16] Gensure RC, Mäkitie O, Barclay C, et al. A novel COL1A1 mutation in infantile cortical hyperostosis (Caffey disease) expands the spectrum of collagen-related disorders. J Clin Invest 2005;115:1250–7.

[17] Bechmann LP, Gastaldelli A, Vetter D, et al. Glucokinase links Krüppel-like factor 6 to the regulation of hepatic insulin sensitivity in nonalcoholic fatty liver disease. Hepatology 2012;55:1083–93.

[18] Romos S, Kožlátnia J, Xing C, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 2008;40:1461–3.

[19] Gallego-Durán R, Gallego P, et al. Unraveling the pathogenesis of fatty liver disease: patatin-like phospholipase domain-containing 3 protein. Curr Opin Lipidol 2010;21:247–52.

[20] Mehta MB, Shewale SV, Sequeira RN, et al. Hepatic protein phosphatase 1 regulatory subunit 3B (PPP1R3B) promotes hepatic glycogen synthesis and thereby regulates fasting energy homeostasis. J Biol Chem 2017;292:10444–54.

[21] Stender S, Smagris E, Lauridsen BK, et al. Relationship between genetic variants to improve risk prediction for NAFLD and its progression to cirrhosis: a proof of concept study. Can J Gastroenterol 2018;2018:7564835.

[22] Lee VN, Hsu-Doran I, Baroni MG, et al. Candidate genes investigation for severe nonalcoholic fatty liver disease based on bioinformatics analysis. Medicine (Baltimore) 2017;96:e7743.

[23] Narla G, Heath KE, Reeves HZ, et al. KL6, a candidate tumor suppressor gene mutated in prostate cancer. Nature 2002;415:530–4.

[24] Berlanga A, Guiu-Jurado E, Porras JA, et al. Molecular pathways in non-alcoholic fatty liver disease. Clin Exp Gastroenterol 2014;7:221–39.

[25] Yu F, Jiang Z, Chen B, et al. NEAT1 accelerates the progression of liver carcinoma using real-time quantitative RT-PCR. BMC Cancer 2013;13:90.

[26] Hallsworth K, Thoma C, Moore S, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002;415:530–6.

[27] Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and treatment of nonalcoholic fatty liver disease based on bioinformatics analysis. J Hepatol 2016;64:1388–90.

[28] Speliotes EK, Yue-Armstrong LM, Wu J, et al. Genome-wide association studies identify a novel adiposity-regulating variant at MTCH2. Nat Genet 2014;46:352–6.

[29] Di Stasi S, Huguet D, Mariani M, et al. Variants associated with nonalcoholic fatty liver disease that have distinct effects on metabolic traits. PLoS Genet 2014;10:e1004323.

[30] Eguchi T, Tsuchida S, Yamauchi-Takihara K, et al. A novel single-nucleotide polymorphism in the gene encoding liver-enriched transcription factor 3 in humans is associated with nonalcoholic fatty liver disease. J Hepatol 2013;59:309–12.

[31] Vervoort CL, Schuuring E, van de Lisdonk E, et al. A new class of adipose tissue markers that predict overall survival in hepatocellular carcinoma. Hepatology 2011;54:1443–52.

[32] Salz WB. Nonalcoholic fatty liver disease (NAFLD): a comprehensive review. J Insur Med 2004;36:27–41.

[33] Erickson SK. Nonalcoholic fatty liver disease. J Lipid Res 2009;50(Suppl):S412–6.

[34] Bahtlawork K, Thoma C, Moore S, et al. Non-alcoholic fatty liver disease is associated with higher levels of objectively measured sedentary behaviour and lower levels of physical activity than matched healthy controls. Frontline Gastroenterol 2015;6:64–81.

[35] Mazziola N, Ricciardi-LM, Mazzotti A, et al. The role of medications for the management of patients with NAFLD. Clin Liver Dis 2014;18:73–89.

[36] Tsibova E, Bayen-Tigges I, Gores JG, et al. Meta-analysis method for discovering genetic variants to improve risk prediction for NAFLD and its progression to cirrhosis: a proof of concept study. Can J Gastroenterol 2018;2018:7564835.