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

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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).[1] 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).[6–8] To date, there is no effective treatment for the advanced stages of NAFLD[1,6,9]; 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

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The datasets generated during and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

The data that support the findings of this study are available from a third party, but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are available from the authors upon reasonable request and with permission of the third party.

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(*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.[15,20,21] 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.[6] 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/m^2) 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 $\geq$ 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 and exclusion criteria.

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

[a] 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.
[b] 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, Waltham, MA). The PCR conditions were as follows – an initial denaturation at 95.0 °C for 10 minutes, followed by 40 cycles of denaturation at 95.0 °C for 15 seconds and annealing or extension at 60.0 °C for 1 minute. All reactions were performed in triplicate. *HMBS* was used as the reference gene, and the relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ equation.

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 $5{,}000 \times g$ 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. OKCD01974; 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 ampilicon size.

Gene	Gene type	PCR primers (5'→ 3')	Product size
HMBS	Reference gene	Forward: ACCCACAGTTGGTAGGCATC Reverse: AAACCAGTTAATGGGCATCG	194 bp
PNPLA3	Candidate gene for steatosis stage	Forward: GAAGGGATGGATCCTGAGGT Reverse: GGTGTCCAGGATGCTCTCAT	197 bp
PPP1R3B	Candidate gene for steatosis stage	Forward: TGCCTTGAGAACTGTGTGCT Reverse: TGTCTGAACCGGCATAAGTG	216 bp
COL1A1	Candidate gene for fibrosis stage	Forward: TGGTTTCGACTTCAGCTTCC Reverse: CTCTTGAGGGTGGTGTCCA	166 bp
KLF6	Candidate gene for fibrosis stage	Forward: CCTCCCTGAATGCATCAAAT Reverse: AGTTTGTCGTTGTGCAGGTG	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.

Table 3

Parameters assessed for the study population.

Parameter	Control group		Steatosis group		Fibrosis group		*P* value
	Mean	SD	Mean	SD	Mean	SD	
Weight (kg)	118.71	23.82	124.77	16.69	122.06	22.40	.854
Height (cm)	162.22	4.94	169.64	10.36	164.63	10.18	.250
BMI (kg/m^2)	44.94	8.77	43.19	1.83	45.37	9.32	.848
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	55.25	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*
Hemoglobin (g/dL)	12.96	1.51	12.13	3.88	14.00	2.09	.381
Total WBC (per mcL)	7.87	1.51	8.49	3.47	10.41	3.05	.195
WBC_neutrophils (×10/l)	3.94	1.74	6.10	3.36	6.36	2.73	.248
Platelet count (×10^3/μL)	296.11	56.76	301.13	79.93	244.00	51.92	.159
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	5.06	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.

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

The mean age of the study participants was 30 years old; their mean BMI was 44.5 kg/m^2 (morbid/severe obesity), and their mean waist circumference was 126.51 cm (large). In the control group, the age ranged from 19 to 60 years. Control group participants were morbidly obese, but did not have NAFLD. Most of the control participants had an active lifestyle (90–150 minutes of exercise weekly) and they all had normal liver enzymes levels (Alanine aminotransferase mean 32.56 ± 4.59 U/L; Aspartate aminotransferase mean 14.7 ± 3.71 U/L; gamma-glutamyltransferase 24.00 ± 4.78 U/L), and normal lipid panel (high-density lipoproteins mean 1.2 ± 0.39 mmol/dL; low-density lipoproteins mean 2.89 ± 0.74 mmol/dL; Cholesterol mean 4.4 ± 0.77 mmol/dL; triglycerides mean 0.88 ± 0.32 mmol/dL).

The liver enzyme, alanine aminotransferase (ALT), was higher in the steatosis group (mean 73.38 ± 35.64 U/L), and gamma-glutamyltransferase (GGT) levels were within the normal range for all participants; aspartate transaminase (AST) levels were slightly high in fibrosis stage of NAFLD, with a mean value of 40 unit/L (±43.97). Lipid profile showed both cholesterol and high-density lipoproteins (HDL) to be within the normal range for all participants. Triglycerides (TG) levels were higher in patients with fibrosis-stage NAFLD (mean 2.47 ± 1.81 mmol/L, and $P < .03$), and the level of low-density lipoproteins (LDL) was high in patients with steatosis-stage NAFLD (mean 3.42 ± 0.97 mmol/L). Other parameters, such as platelet count, hemoglobin, and creatinine, were within the normal range for all participants (Table 3). More than half (62.5%) of the patients with fibrosis complained of fatigue, and 25% presented with jaundice.

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 ($P < .04$). 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* ($P = .03$) and *COL1A1* in the fibrosis stage ($P = .03$), in obese patients with NAFLD. In the control group, there was a positive correlation between *PNPLA3* protein expression and levels of hemoglobin ($r = 0.78$, $P = .02$), MCV ($r = 0.72$, $P = .04$), and platelets ($r = 0.798$, $P = .04$). However, there was a negative correlation between the expression of COL1A1 protein and hemoglobin ($r = -0.766$,

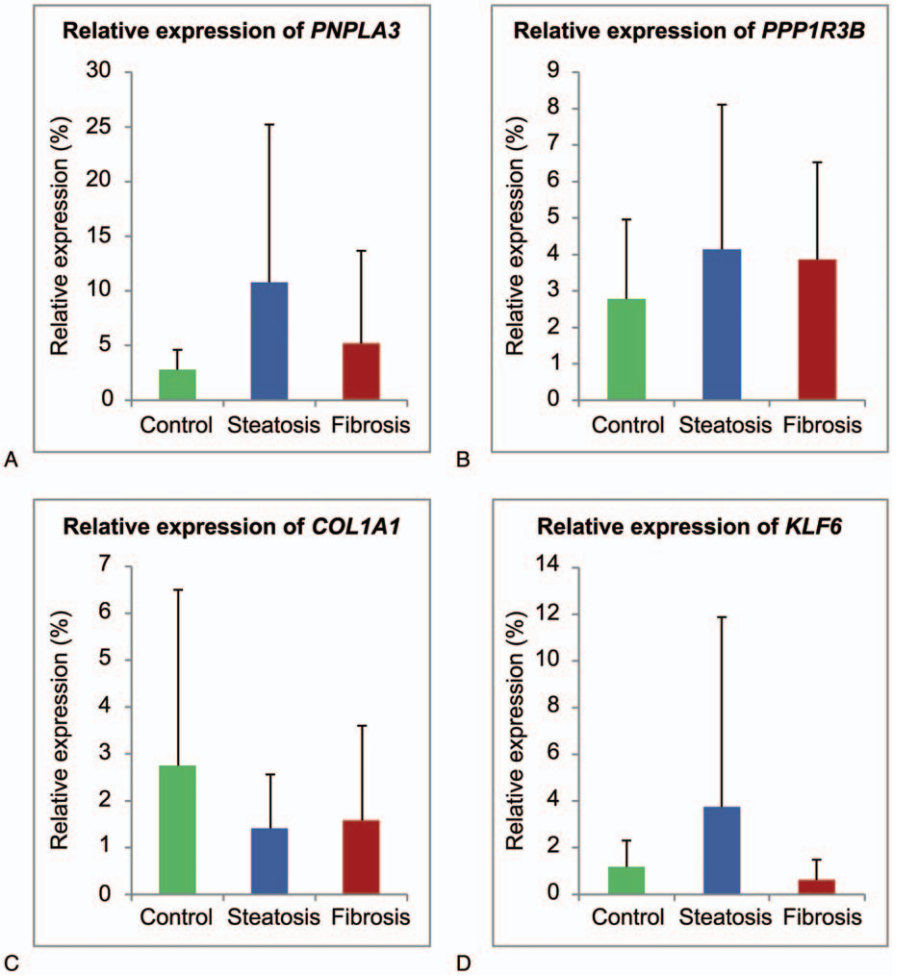

Figure 1. Expression of candidate biomarker genes in non-alcoholic fatty liver disease (NAFLD). Expression of candidate biomarker genes for the steatosis stage of NAFLD: (A) patatin-like phospholipase domain-containing protein 3 and (B) protein phosphatase 1, regulatory subunit 3B, and that for the fibrosis stage of NAFLD: (C) collagen type 1 α 1 and (D) Kruppel-Like Factor 6, determined by real-time quantitative PCR. No significant difference was observed in the expression of genes in the study groups compared to that in the control subjects. Gene expression was determined using the $2^{-\Delta\Delta CT}$ method and the values are expressed as mean ± SD.

$P=.03$) level. In patients with fibrosis, we observed a positive correlation of *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 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 *PNPLA3*, *PPP1R3B*, *COL1A1*, and *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 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 *PNPLA3* protein to be related to advanced NAFLD or fibrosis; which explain the significant expression of *PNPLA3* protein in patients with fibrosis.[33] However, in our study, there was no significant difference in the expression of *PNPLA3*, *PPP1R3B*, *COL1A1*, or *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.[8] Fibrosis is the third stage and the only stage that is unaffected by age or diabetes mellitus (DM).[8] 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

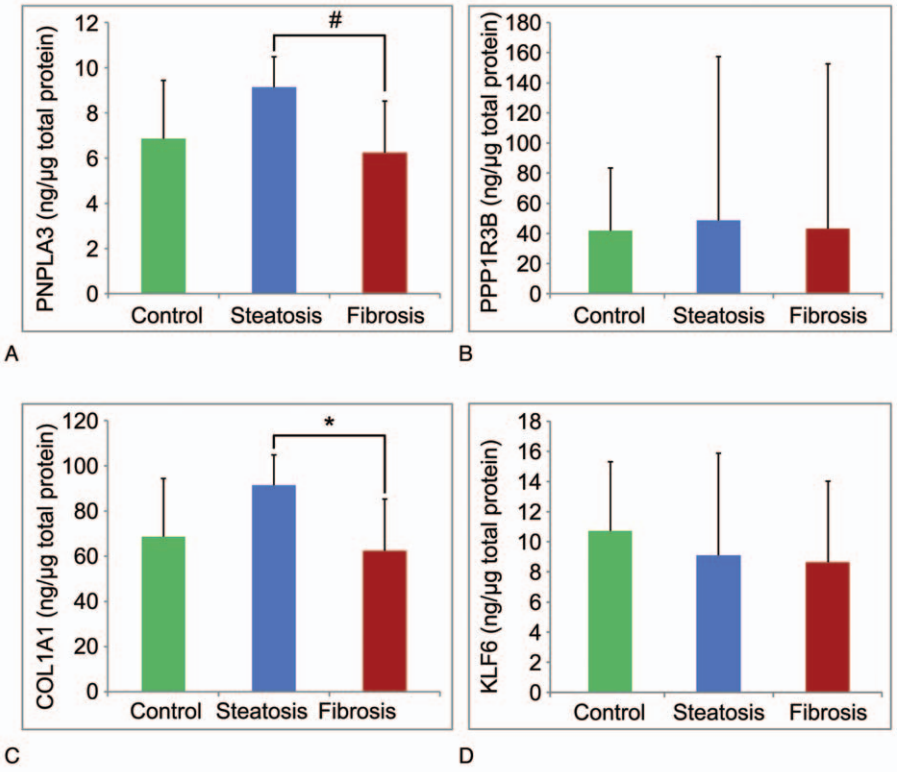

Figure 2. Expression of candidate biomarker proteins for non-alcoholic fatty liver disease (NAFLD) in steatosis and fibrosis stages. Expression of (A) patatin-like phospholipase domain-containing protein 3 protein and (B) protein phosphatase 1, regulatory subunit 3 protein in the liver tissue of patients with NAFLD, determined by enzyme-linked immunosorbent assay. A significant difference was found in the expression of patatin-like phospholipase domain-containing protein 3 protein in the fibrosis stage compared to that in the steatosis stage of NAFLD. The plots show mean ± SEM of the relative expression. $^{\#}P < .04$. Expression of (C) collagen type 1 α 1 and (D) Kruppel-Like Factor 6 proteins in the liver tissue of patients with NAFLD, determined by enzyme-linked immunosorbent assay. There was a significant difference in the expression of collagen type 1 α 1 protein in the fibrosis stage of NAFLD compared to that in the steatosis stage. The plots show mean ± SEM of the relative expression. $^{*}P < .04$.

accumulation of lipid droplets in more than 5% of hepatocytes.[45] There are 3 types of HS (or fatty liver) –

(i) microvesicular,
(ii) macrovesicular, and
(iii) mixed steatosis.[8]

Mixed steatosis is the most common in patients with NAFLD, in which both micro- and macro-vesicular steatosis are present.[37]

The fibrosis stage is considered a progressive stage in NAFLD.[4] Hepatic fibrosis is a result of 2 types of insults to hepatocytes – cell injury and necro-inflammation.[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.[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.[48]

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

Table 4

Correlation analysis of selected genes and assessed parameters.

Stage	Investigated biomarker	Related parameter	Correlation (r^2)
Steatosis	*PPP1R3B*	Body mass index (BMI)	0.81
	PNPLA3	Gamma-glutamyl transferase (GGT)	0.88
		Triglycerides	0.96
	COL1A1	Waist circumference	−0.83
	KLF6		−0.86
	COL1A1	Low-density lipoproteins (LDL)	0.77
	KLF6	Creatinine	0.83
		urea	0.54 (P=.055; positive trend)
Fibrosis	*PNPLA3*	Potassium	0.85

COL1A1 = collagen type 1 α 1, *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.[51] Genetics plays a critical role in NAFLD, since genetic variations impact disease progression in patients with NAFLD.[3] Several studies have investigated the gene expression-based staging of NAFLD to better understand its progression and to identify effective treatments.[52] To date, 2 genes have been identified in NAFLD –

(i) Patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) by genome-wide association study (GWAS) and
(ii) Transmembrane 6 superfamily member 2 (*TM6SF2*) by exome-wide association study (EWAS).[53]

Single nucleotide polymorphisms (SNP) are single nucleotide substitutions in DNA, which generally alter the expression/activity of a specific gene or the function of the proteins.[54] The *PNPLA3* variant, rs738409, is observed in 50% of the general population, and the *TM6SF2* variant, rs58542926, is observed in 10% of the population.[55,56] These 2 SNPs have been reported to increase the risk of fibrosis in NAFLD by 40% to 80%.[55] Further, the *PNPLA3* variant, rs738409, increases the risk of HCC.[56] There was also a significant association between *PNPLA3* gene expression and elevated hepatic fat content in fatty liver. Genetic variations of *PNPLA3* is reported to be associated with obesity, insulin resistance (in hepatic and adipose tissues), and steatosis in NAFLD across 3 different ethnicities – Hispanic, American-European, and Africans.[18] Up-regulation of *PPP1R3B* expression is related to increased glycogen storage in the liver and reduced hepatic fat content in ultrasonography, though not in histology.[20] *PPP1R3B* is suggested to possibly play a protective role against fibrosis and HCC in NAFLD since it can modulate lipid metabolism.[20] However, there is insufficient evidence on *PPP1R3B* expression in the steatosis stage of NAFLD.

Herein, we did not observe any significant difference in the expression of *COL1A1* and *KLF6* genes in NAFLD; however, a significant increase in the expression of COL1A1 protein in the steatosis stage of NAFLD, was observed compared to that in the control group ($P < .03$) and fibrosis group ($P < .01$). Furthermore, the mean COL1A1 protein expression was higher by 30% in the steatosis group compared to that in the other 2 groups.

Moreover, our results also confirm specific associations between investigated biomarkers and clinical parameters. High BMI was shown to increase the risk of steatosis, since the expression of *PPP1R3B* increases when BMI increases. Hence, obesity is known to be a major risk for NAFLD.[8] Hypertriglycemia is also a major risk factor for steatosis.[8] This study confirmed this, since high TGs in the blood stream were shown to increase the risk of high expression of *PNPLA3*, which eventually increases the risk of HS.[57]

Considering that studies have reported large waist circumference as a risk factor for NAFLD,[9] normal waist circumference then contributes toward the prevention of hepatic fibrosis development in NAFLD. As confirmed by the results of this study, there is a negative association between waist circumference and 2 potential fibrogenic genes, including *COL1A1* and *KLF6*. There are inadequate data about the relationship between waist circumference and the 2 genes in hepatic fibrosis. However, waist circumference with COL1A1 has been related to the severity of fibrosis in adipose tissues.[10]

Similar to our previous studies, we again determined that the lipid profile is related to NAFLD, since dyslipidemia increased the risk of both stages of NAFLD (including steatosis and fibrosis).[11,12] High LDL levels in blood also increased the risk of fibrosis or advanced NAFLD.[12] As shown in our results, high LDL levels elevate the expression of *COL1A1*, which had previously been shown in the case of tendons.[13] 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.[58–60] NAFLD increases the risk of chronic kidney disease (CKD), since studies have shown a strong relationship between biopsy-proven NAFLD and early kidney disease.[14,33]

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.[18,61] 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*).[15]
(3) Potential biomarkers for fibrosis: waist circumference has an inverse association with fibrosis (by lowering the expression of *COL1A1* and *KLF6*).[2,19] 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*).[20]

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.[21] 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.

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