

Original Article

Serum FGF21 as a predictor of hepatic steatosis severity in non-alcoholic fatty liver disease: an integrated molecular and clinical analysis

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Abstract: The aim of this paper is to assess serum fibroblast growth factor 21 (FGF21) concentration as a potential biomarker for hepatic steatosis severity in Non-Alcoholic Fatty Liver Disease (NAFLD) through an integrated molecular and clinical approach. This retrospective study assessed 120 NAFLD patients and collected clinical, biochemical, genomic, transcriptomic, and proteomic data. Integrated molecular analyses revealed that key lipid metabolism genes (PNPLA3, TM6SF2, GCKR) were significantly enriched in patients with severe steatosis, accompanied by upregulation of FGF21-related transcriptional pathways (PPAR α and ATF4 signaling). Proteomic profiling demonstrated increased expression of lipid metabolism-associated proteins, while metabolomic analysis indicated elevated free fatty acids and disrupted ketone metabolism. These molecular alterations were strongly correlated with circulating FGF21 levels and hepatic fat fraction. Among all the tested parameters Serum FGF21 was identified as the most accurate predictor of severe hepatic steatosis with its concentration rising progressively from mild to severe conditions. The study also confirmed associations between metabolic disorders, markers of hepatic injury, and inflammation with disease progression. Multivariate logistic regression identified independent predictors, including BMI/obesity-related parameters, insulin resistance, indices of hepatic fibrosis, and alanine transaminase. Receiver operating characteristic (ROC) curve analysis further demonstrated that FGF21 exhibited good sensitivity and specificity for the discrimination of mild, moderate, and severe hepatic steatosis, suggesting its potential as a non-invasive biomarker for disease monitoring.

Keywords: Fibroblast growth factor 21, hepatic steatosis, non-alcoholic fatty liver disease, biomarkers, diagnostic performance

Introduction

Non-alcoholic fatty liver disease (NAFLD) has emerged as the most common chronic liver disorder worldwide and currently affects approximately 25-30% of the global population. The increasing prevalence of NAFLD parallels the rising burden of obesity, metabolic syndrome, insulin resistance, and type 2 diabetes mellitus, making it a major global public health concern. NAFLD encompasses a broad spectrum of hepatic abnormalities ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), progressive fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). In addition to

liver-related morbidity, NAFLD is strongly associated with several extrahepatic complications, including cardiovascular disease and chronic kidney disease, highlighting its systemic metabolic nature [1-3].

Despite substantial advances in understanding NAFLD pathogenesis, early identification of patients at risk for progressive hepatic steatosis and advanced disease remains clinically challenging. Liver biopsy remains the reference standard for diagnosis and staging; however, its invasive nature, sampling variability, procedural risks, and limited suitability for routine screening restrict widespread clinical applica-

tion. Imaging modalities such as magnetic resonance imaging-proton density fat fraction (MRI-PDFF), controlled attenuation parameter (CAP), and transient elastography provide useful non-invasive assessment, although they may be limited by cost, accessibility, and reduced sensitivity in detecting early metabolic alterations [4, 5]. Consequently, there is increasing interest in identifying reliable circulating biomarkers that can facilitate non-invasive diagnosis, disease stratification, and monitoring of NAFLD progression. Fibroblast growth factor 21 (FGF21) is an endocrine member of the fibroblast growth factor superfamily that plays a central role in regulating glucose metabolism, lipid homeostasis, fatty acid oxidation, ketogenesis, and energy balance. FGF21 is predominantly synthesized in the liver, and it exerts metabolic effects through fibroblast growth factor receptors in the presence of the β -Klotho co-receptor in metabolically active tissues including adipose tissue, skeletal muscle, and liver. Under physiological conditions, circulating FGF21 levels remain relatively low; however, its expression is induced during fasting and metabolic stress and circulating concentrations are frequently elevated in obesity, insulin resistance, and fatty liver disease [6-8]. Clinical studies have demonstrated that elevated serum FGF21 concentrations are associated with hepatic steatosis and metabolic dysfunction, suggesting that FGF21 may serve as a compensatory metabolic stress response factor during NAFLD progression [9-11].

NAFLD development is multifactorial and involves complex interactions between genetic susceptibility, metabolic dysregulation, inflammation, oxidative stress, and environmental influences. Hepatic steatosis primarily results from excessive triglyceride accumulation within hepatocytes due to increased lipogenesis, enhanced free fatty acid influx, impaired fatty acid oxidation, and insulin resistance. Progressive lipid accumulation promotes lipotoxicity, oxidative stress, mitochondrial dysfunction, and inflammatory signaling pathways that contribute to the transition from simple steatosis to NASH and advanced fibrosis [12, 13]. Furthermore, several genetic polymorphisms, including variants in patatin-like phospholipase domain-containing protein 3 (PNPLA3), transmembrane 6 superfamily member 2 (TM6SF2),

glucokinase regulatory protein (GCKR), and hydroxysteroid 17-beta dehydrogenase 13 (HSD17B13), have been implicated in susceptibility to hepatic fat accumulation and disease severity [14, 15]. Recent advances in molecular profiling technologies have improved understanding of the biological mechanisms underlying NAFLD progression. Alterations in lipid metabolism, inflammatory signaling, oxidative stress pathways, amino acid metabolism, and gut microbiome composition have all been associated with worsening hepatic steatosis. In particular, gut dysbiosis, altered short-chain fatty acid production, and endotoxin-related inflammatory signaling may contribute to hepatic injury and metabolic dysfunction through the gut-liver axis [16, 17]. In addition, transcriptional regulators such as peroxisome proliferator-activated receptor alpha (PPAR α) and activating transcription factor 4 (ATF4) have been identified as important modulators of FGF21 expression during metabolic stress conditions [18, 19]. Emerging evidence further suggests that circulating FGF21 levels correlate with hepatic fat content and metabolic abnormalities, supporting its potential utility as a non-invasive biomarker for NAFLD assessment [9, 10, 20-22].

Several studies have demonstrated significant increases in serum FGF21 concentrations among patients with moderate and severe hepatic steatosis, with positive correlations observed between FGF21 levels and MRI-PDFF, CAP scores, hepatic fat fraction, and metabolic dysfunction [9, 20-22]. Furthermore, alterations in circulating free fatty acids, acylcarnitines, ketone body metabolism, inflammatory mediators, and microbiome-derived metabolites have been linked with elevated FGF21 concentrations, indicating that FGF21 may reflect systemic metabolic stress and lipotoxicity associated with NAFLD progression [13, 16, 23]. These findings suggest that integrated molecular and metabolic analyses may provide additional insights into the pathophysiological role of FGF21 in hepatic steatosis progression.

Therefore, the present study aimed to evaluate serum FGF21 as a predictive biomarker of hepatic steatosis severity in patients with NAFLD and to investigate its association with integrated molecular, metabolic, inflammatory, and microbiome-related alterations. Addi-

tionally, we assessed the diagnostic performance of serum FGF21 and explored its relationship with clinical, biochemical, and molecular parameters associated with disease progression.

Materials and methods

Study design and participants

This retrospective study assessed 120 participants aged between 18 and 75 years who fulfilled the clinical and imaging criteria for NAFLD. Participants were consecutively recruited from the outpatient hepatology and metabolic disease clinics of The First Affiliated Hospital of Bengbu Medical University between January 2023 and December 2024.

NAFLD diagnosis was established based on radiological evidence of hepatic steatosis in the absence of secondary causes of liver fat accumulation. Exclusion criteria: (1) daily alcohol consumption > 20 g/day for women and > 30 g/day for men; (2) viral hepatitis B or C infection; (3) autoimmune hepatitis; (4) Wilson's disease; (5) pregnancy; (6) history of malignancy; (7) significant weight loss (> 5% body weight) within the preceding 3 months; (8) use of hepatotoxic medications; and (9) current treatment with drugs known to significantly affect lipid or glucose metabolism.

The study protocol was approved by the Institutional Review Board of The First Affiliated Hospital of Bengbu Medical University (Approval No. 2023YJS098) and was conducted in accordance with the Declaration of Helsinki.

Clinical and laboratory data collection

Demographic and clinical information including age, sex, smoking history, alcohol intake, dietary habits, physical activity, and medical history of diabetes mellitus and hypertension were collected using structured questionnaires and medical record review. Physical activity levels were assessed using the International Physical Activity Questionnaire (IPAQ).

Anthropometric measurements were obtained using standardized procedures. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m²). Waist circumference (WC) was measured midway between the lower rib margin and the

iliac crest. Body fat percentage was determined using bioelectrical impedance analysis.

Following overnight fasting, venous blood samples were collected for biochemical analyses including:

(1) Liver function parameters: alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), and alkaline phosphatase (ALP); (2) Lipid profile: total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C); (3) Glycemic parameters: fasting blood glucose (FBG), glycated hemoglobin (HbA1c), and fasting insulin; (4) Inflammatory markers: high-sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α); (5) Oxidative stress markers: malondialdehyde (MDA) and total antioxidant capacity (TAC).

Insulin resistance was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR).

Assessment of hepatic steatosis

Hepatic steatosis severity was evaluated using multiple imaging modalities.

Ultrasonography

Abdominal ultrasonography was performed by an experienced radiologist blinded to laboratory findings. Hepatic steatosis was graded as mild, moderate, or severe based on hepatic echogenicity and hepatorenal contrast.

Magnetic resonance imaging-proton density fat fraction (MRI-PDFF)

MRI-PDFF was used for quantitative assessment of hepatic fat content.

Transient elastography

Transient elastography (FibroScan®, Echosens, Paris, France) was performed to evaluate liver stiffness and steatosis. Liver stiffness measurement (LSM) was used to assess fibrosis, while CAP values were used to quantify hepatic steatosis.

Measurement of serum FGF21

Serum FGF21 concentrations were measured using a commercially available high-sensitivity

enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems DF2100, 31.3-2,000 pg/mL). The assay detection range, analytical sensitivity, and intra-assay/inter-assay coefficients of variation were determined according to the manufacturer's protocol.

Blood samples were centrifuged immediately after collection and serum aliquots were stored at -80°C until analysis. All assays were performed in duplicate and analyzed in a single batch to minimize inter-assay variability.

Integrated molecular and microbiome analyses

Genomic analysis: Genotyping of NAFLD-associated polymorphisms in PNPLA3, TM6SF2, and GCKR genes was performed using polymerase chain reaction (PCR) followed by Sanger sequencing.

Epigenetic analysis: Genomic DNA extracted from peripheral blood samples underwent bisulfite conversion for evaluation of DNA methylation status in promoter regions of genes associated with lipid metabolism and inflammatory signaling.

Transcriptomic analysis: Total RNA was isolated from peripheral whole blood samples using the TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quality and integrity were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and only samples with RNA Integrity Number (RIN) ≥ 7.0 were included for downstream analyses.

RNA-seq libraries were prepared using the Illumina TruSeq Stranded mRNA Library Preparation Kit (Illumina, San Diego, CA, USA). Sequencing was performed on the Illumina NovaSeq 6000 platform generating paired-end 150-bp reads with an average sequencing depth of approximately 40 million reads per sample. Raw sequencing data underwent quality assessment using FastQC (version 0.11.9). Adapter trimming and low-quality read removal were performed using Trimmomatic (version 0.39). Clean reads were aligned to the human reference genome (GRCh38) using STAR aligner (version 2.7.10a). Gene expression quantification was performed using featureCounts (Subread package version 2.0.3). Differen-

tial expression analysis was conducted using DESeq2 (version 1.38.0), and genes with false discovery rate (FDR)-adjusted *P*-values < 0.05 were considered significantly differentially expressed.

Proteomic analysis: Proteomic profiling was performed using ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS). Protein extraction was carried out using radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors.

Protein digestion was performed using sequencing-grade modified trypsin (Promega, Madison, WI, USA). Peptide separation was conducted using an EASY-nLC 1200 system (Thermo Fisher Scientific, Bremen, Germany) coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific).

Mass spectra were acquired in data-dependent acquisition mode with full MS scans obtained at 60,000 resolutions followed by higher-energy collisional dissociation (HCD) fragmentation.

Protein identification and quantification were performed using MaxQuant software (version 2.1.0) against the UniProt Homo sapiens database. False discovery rates were controlled at < 1% at both peptide and protein levels. Differentially expressed proteins were defined using a fold-change threshold ≥ 1.5 and FDR-adjusted *P*-value < 0.05.

Metabolomic analysis: Gas chromatography-mass spectrometry (GC-MS) was used for untargeted metabolomic profiling. Protein precipitation was performed with methanol and the protein precipitate was derivatized with methoxyamine hydrochloride in pyridine and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) before analysis. The separation was done on an Agilent 8890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) with a DB-5MS (30 m × 0.25 mm × 0.25 μm) capillary column and a mass selective detector (Agilent 5977B). Mass spectra were obtained using electron impact ionization at 70 eV and 50 to 600 *m/z*. The metabolite identification was carried out by searching the NIST 2023 Mass Spectral Library and Human Metabolome Database (HMDB). Metabolite quan-

tification, peak alignment and normalization were performed with MetaboAnalyst version 6.0. Metabolites showing FDR adjusted *P*-values < 0.05 were deemed to be significantly altered.

Gut microbiome analysis: The 16S ribosomal RNA gene sequencing of the V3-V4 hyper-variable region was used to characterize the composition of the gut microbiome. The DNA was isolated from the fecal sample with the “QIAamp Fast DNA Stool Mini Kit” (Qiagen, Hilden, Germany). Library preparation and sequencing were done with universal primers 341F and 806R, and sequencing was done on the Illumina MiSeq platform using paired end reads of 2 × 300 bp. QIIME2 (version 2024.2) was used for sequence processing, quality filtering, operational taxonomic unit (OTU) clustering and taxonomic assignment. The taxonomic classification was done against the database SILVA 138. Firmicutes/Bacteroidetes ratio, alpha diversity, and beta diversity were then assessed.

Multi-layer molecular data integration

Integrated molecular analysis was performed using R software (Version 4.3.1). Pathway enrichment analysis of the differentially expressed genes, proteins, metabolites and microbiome features was performed via the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) databases. Spearman correlation analyses were performed to create correlation networks of serum FGF21 levels with molecular characteristics. Multiple-testing correction was performed using the Benjamini-Hochberg false discovery rate method, and adjusted *P*-values < 0.05 were considered statistically significant.

Dietary assessment

Dietary intake was assessed using a face-to-face 24-hour dietary recall interview combined with a validated food frequency questionnaire (FFQ). Daily macro- and micronutrient intake was estimated using standard nutritional databases.

Statistical analysis

Statistical analyses were performed using SPSS version 25.0 (IBM Corp., Armonk, NY, USA). Data normality was assessed using the

Shapiro-Wilk test. Continuous variables were expressed as mean ± standard deviation (SD) for normally distributed data and median with interquartile range (IQR) for non-normally distributed variables. Categorical variables were presented as frequencies and percentages.

Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for normally distributed continuous variables. Non-normally distributed variables were analyzed using the Kruskal-Wallis test. Chi-square test was used for categorical variables.

Correlations between serum FGF21 levels and clinical, biochemical, imaging, and molecular parameters were evaluated using Pearson or Spearman correlation analyses, as appropriate.

Multivariate logistic regression analyses were performed to identify independent predictors of severe hepatic steatosis after adjustment for potential confounders including age, sex, BMI, and HOMA-IR. Continuous variables included in regression analyses were standardized prior to modeling. Odds ratios (ORs) were expressed per standard deviation increase with corresponding 95% confidence intervals (CIs).

Receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic performance of serum FGF21 in identifying moderate-to-severe hepatic steatosis. A two-tailed *P*-value < 0.05 was considered statistically significant.

Sample size estimation and statistical power

A priori sample size estimation was performed prior to study initiation to ensure adequate statistical power for multivariate regression analyses and integrated molecular comparisons. Based on previous studies evaluating serum FGF21 and hepatic steatosis severity in NAFLD populations, a moderate effect size (Cohen's $f^2 = 0.15$) was assumed for multivariable modeling. Using an alpha level of 0.05 and statistical power of 80%, the minimum required sample size for logistic regression analysis including up to 10 predictor variables was estimated to be approximately 110 participants. Therefore, a total sample size of 120 participants was considered adequate to sa-

Table 1. Demographic, clinical, lifestyle, and dietary factors by hepatic steatosis severity

Variable	Mild Steatosis (n = 40)	Moderate Steatosis (n = 40)	Severe Steatosis (n = 40)	P-value
Age (years)	42.6 ± 10.41	45.8 ± 11.12	47.2 ± 9.82	0.118
Male sex, n (%)	21 (52.51)	24 (60.03)	27 (67.53)	0.421
BMI (kg/m ²)	25.12 ± 2.32	28.4 ± 3.12 ^a	32.1 ± 3.90 ^{a,b}	< 0.001
Waist circumference (cm)	87.31 ± 8.62	95.7 ± 9.13 ^a	104.8 ± 10.72 ^{a,b}	< 0.001
Body fat percentage (%)	27.62 ± 5.12	32.8 ± 5.91 ^a	38.4 ± 6.32 ^{a,b}	< 0.001
Daily caloric intake (kcal/day)	1864 ± 312	2215 ± 356 ^a	2587 ± 402 ^{a,b}	< 0.001
Physical activity (MET-min/week)	1825 (1450-2230)	1410 (1180-1760) ^a	980 (740-1350) ^{a,b}	< 0.001
Current smokers, n (%)	9 (22.52)	12 (30.00)	15 (37.51)	0.331
Type 2 diabetes mellitus, n (%)	8 (20.03)	14 (35.00)	21 (52.51) ^b	0.006
Hypertension, n (%)	10 (25.02)	16 (40.00)	23 (57.52) ^b	0.009
Fasting blood glucose (mg/dL)	94.71 ± 11.21	108.52 ± 14.6 ^a	124.1 ± 18.35 ^{a,b}	< 0.001
HbA1c (%)	5.62 ± 0.52	6.12 ± 0.7 ^a	6.8 ± 0.91 ^{a,b}	< 0.001
Fasting insulin (μIU/mL)	9.22 ± 3.41	14.81 ± 5.1 ^a	21.6 ± 6.72 ^{a,b}	< 0.001
HOMA-IR	2.12 ± 0.83	4.02 ± 1.32 ^a	6.7 ± 2.22 ^{a,b}	< 0.001

Footnotes: Data are presented as Mean ± SD or n (%), as appropriate. P-values were calculated using one-way ANOVA or Chi-square tests. a = Significant compared with mild steatosis group (P < 0.05). b = Significant compared with moderate steatosis group (P < 0.05). BMI: body mass index; HOMA-IR: homeostasis model assessment of insulin resistance; MET: metabolic equivalent task.

tisfy statistical power requirements while accounting for potential missing data and subgroup comparisons.

For transcriptomic, proteomic, and metabolomic analyses, the sample size was additionally considered sufficient to detect biologically meaningful differential molecular expression with acceptable false discovery rate (FDR) correction. Statistical power calculations and regression modeling assumptions were evaluated using *GPower software version 3.1* (Heinrich-Heine-University Düsseldorf, Germany). To ensure consistency in data presentation, all continuous measurement variables were reported to two decimal places, whereas statistical parameters including P-values, odds ratios, confidence intervals, correlation coefficients, and receiver operating characteristic (ROC) statistics were reported to three decimal places throughout the manuscript. Statistical significance indicators used in tables were defined uniformly as P < 0.05 (*) and P < 0.01 (**).

Results

Demographic, clinical, lifestyle, and dietary factors by hepatic steatosis severity

The demographic, clinical, lifestyle, and dietary characteristics of participants stratified by hepatic steatosis severity (mild, moderate, and

severe) are presented in **Table 1**. Age showed a slight increase across groups (from 42.6 to 47.2 years), although this difference was not statistically significant (P = 0.118). BMI increased significantly with disease severity, from 25.12 kg/m² in the mild group to 32.1 kg/m² in the severe group (P < 0.001). Similarly, waist circumference increased from 87.31 cm to 104.8 cm (P < 0.001). Insulin resistance, assessed by HOMA-IR, increased across severity stages (2.12 ± 0.83 in mild, 4.02 ± 1.32 in moderate, and 6.70 ± 2.22 in severe; P < 0.001), indicating progressive deterioration of metabolic homeostasis. Physical activity decreased across severity groups (median 1825, 1410, and 980 MET-min/week in the mild, moderate, and severe groups, respectively; P < 0.001). The proportion of current smokers increased numerically across groups (22.5%, 30.0%, and 37.5%), but the difference was not statistically significant (P = 0.331). Daily caloric intake also increased progressively with steatosis severity, from 1864 ± 312 kcal/day in the mild group to 2215 ± 356 kcal/day in the moderate group and 2587 ± 402 kcal/day in the severe group (P < 0.001).

Biochemical, lipid, and glycemic parameters by hepatic steatosis severity

Serum lipid profiles demonstrated progressive deterioration with increasing hepatic steatosis

Table 2. Biochemical, lipid, and glycemic parameters by hepatic steatosis severity

Variable	Mild Steatosis (n = 40)	Moderate Steatosis (n = 40)	Severe Steatosis (n = 40)	P-value
ALT (U/L)	32.84 ± 11.52	51.45 ± 17.88 ^a	74.68 ± 25.13 ^{a,b}	< 0.001
AST (U/L)	28.66 ± 9.31	39.82 ± 12.74 ^a	57.32 ± 18.49 ^{a,b}	< 0.001
GGT (U/L)	35.49 ± 14.83	52.63 ± 18.98 ^a	78.24 ± 25.61 ^{a,b}	< 0.001
ALP (U/L)	81.33 ± 16.54	92.44 ± 19.66	104.76 ± 21.81 ^{a,b}	0.003
Total cholesterol (mg/dL)	178.61 ± 28.72	201.54 ± 34.22 ^a	224.97 ± 39.11 ^{a,b}	< 0.001
Triglycerides (mg/dL)	132.83 ± 41.62	176.54 ± 54.74 ^a	228.34 ± 70.25 ^{a,b}	< 0.001
HDL-C (mg/dL)	49.21 ± 8.61	43.72 ± 7.84 ^a	37.94 ± 6.98 ^{a,b}	< 0.001
LDL-C (mg/dL)	102.72 ± 24.52	124.63 ± 29.85 ^a	145.37 ± 34.12 ^{a,b}	< 0.001
hs-CRP (mg/L)	2.63 ± 1.12	4.81 ± 1.93 ^a	7.53 ± 2.88 ^{a,b}	< 0.001
IL-6 (pg/mL)	4.21 ± 1.62	7.87 ± 2.44 ^a	12.17 ± 3.73 ^{a,b}	< 0.001
TNF-α (pg/mL)	10.50 ± 3.24	15.44 ± 4.64 ^a	22.33 ± 6.15 ^{a,b}	< 0.001
MDA (nmol/mL)	2.81 ± 0.95	4.56 ± 1.34 ^a	6.76 ± 1.88 ^{a,b}	< 0.001
TAC (mmol/L)	1.42 ± 0.24	1.18 ± 0.20 ^a	0.93 ± 0.18 ^{a,b}	< 0.001
Serum FGF21 (pg/mL)	148.61 ± 52.32	268.95 ± 81.72 ^a	421.56 ± 126.41 ^{a,b}	< 0.001

Footnotes: Data are presented as Mean ± SD or n (%), as appropriate. P-values were calculated using one-way ANOVA or Chi-square tests. a = Significant compared with mild steatosis group (P < 0.05). b = Significant compared with moderate steatosis group (P < 0.05). ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyl transferase; ALP: alkaline phosphatase; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; hs-CRP: high-sensitivity C-reactive protein; IL-6: interleukin-6; TNF-α: tumor necrosis factor-alpha; MDA: malondialdehyde; TAC: total antioxidant capacity; FGF21: fibroblast growth factor 21.

severity. Total cholesterol levels increased significantly from 178.61 ± 28.72 mg/dL in the mild steatosis group to 201.54 ± 34.22 mg/dL in the moderate steatosis group and 224.97 ± 39.11 mg/dL in the severe steatosis group (P < 0.001). Similarly, LDL-C and triglyceride concentrations increased significantly across disease severity categories, whereas HDL-C levels progressively declined. These findings indicate worsening dyslipidemia accompanying hepatic fat accumulation and metabolic dysfunction. Glycemic parameters also increased. Fasting blood glucose increased from 94.71 to 124.1 mg/dL, HbA1c from 5.62% to 6.8%, and fasting insulin from 9.22 to 21.6 µIU/mL (all P < 0.001), indicating glycemic deterioration. Fasting insulin levels increased more than two-fold (from 8.5 µIU/mL to 16.4 µIU/mL), indicating declining insulin sensitivity (P < 0.001) (Table 2).

Inflammatory and oxidative stress markers by hepatic steatosis severity

Inflammatory and oxidative stress markers increased progressively with the severity of hepatic steatosis. Specifically, hs-CRP levels increased from 2.5 mg/L in mild steatosis to

5.6 mg/L in severe steatosis (P < 0.001). Similarly, pro-inflammatory cytokines, including IL-6 and TNF-α, were significantly elevated with disease severity (IL-6: from 5.2 to 9.6 pg/mL; TNF-α: from 6.5 to 12.6 pg/mL; both P < 0.001). Oxidative stress markers showed a similar trend. Levels of oxidative stress increased from 2.1 nmol/mL in mild cases to 4.5 nmol/mL in severe cases (P < 0.001). In contrast, antioxidant defense capacity was significantly reduced, as total antioxidant capacity (TAC) decreased from 2.9 to 1.8 mmol/L and glutathione levels declined from 4.2 to 2.2 µmol/L (both P < 0.001), indicating impaired antioxidant defense with increasing disease severity. Adipokines also exhibited a clear severity-dependent pattern. Leptin levels increased from 15.4 ng/mL in the mild group to 31.6 ng/mL in the severe group, whereas adiponectin levels decreased from 8.5 µg/mL to 4.2 µg/mL across the same severity groups, suggesting a shift toward a pro-inflammatory adipokine profile. Additionally, oxLDL levels increased from 45.6 mg/dL to 85.4 mg/dL (P < 0.001), further indicating enhanced oxidative stress in severe steatosis. The details are shown in Table 3.

Table 3. Inflammatory and oxidative stress markers by hepatic steatosis severity

Parameter	Mild (n = 40)	Moderate (n = 40)	Severe (n = 40)	F-value	P-value
hs-CRP (mg/L)	2.56 ± 1.03	3.85 ± 1.23	5.63 ± 1.50	29.71	< 0.001**
IL-6 (pg/mL)	5.25 ± 1.83	7.41 ± 2.14	9.61 ± 2.55	24.53	< 0.001**
TNF-α (pg/mL)	6.55 ± 2.04	9.38 ± 2.75	12.64 ± 3.16	34.84	< 0.001**
MDA (nmol/mL)	2.17 ± 0.62	3.26 ± 0.86	4.53 ± 1.07	41.92	< 0.001**
TAC (mmol/L)	2.91 ± 0.76	2.42 ± 0.68	1.86 ± 0.57	16.75	< 0.001**
Leptin (ng/mL)	15.42 ± 4.57	22.88 ± 6.22	31.62 ± 7.87	30.21	< 0.001**
Adiponectin (µg/mL)	8.53 ± 2.38	6.81 ± 1.91	4.21 ± 1.12	25.12	< 0.001**
Oxidized LDL (mg/dL)	45.64 ± 10.21	65.27 ± 12.83	85.42 ± 15.31	50.34	< 0.001**
Glutathione (µmol/L)	4.23 ± 0.82	3.14 ± 0.73	2.23 ± 0.53	35.78	< 0.001**

Data are presented as Mean ± SD or n (%), as appropriate. P-values were calculated using one-way ANOVA or Chi-square tests. *P < 0.05; **P < 0.01. hs-CRP: high-sensitivity C-reactive protein; IL-6: interleukin-6; TNF-α: tumor necrosis factor-alpha; MDA: malondialdehyde; TAC: total antioxidant capacity; LDL: low-density lipoprotein.

Table 4. Liver stiffness, imaging parameters, and related biochemical markers by hepatic steatosis severity

Parameter	Mild (n = 40)	Moderate (n = 40)	Severe (n = 40)	F-value	P-value
Liver Stiffness (kPa)	5.21 ± 0.95	7.81 ± 1.24	11.63 ± 2.45	65.28	< 0.001**
CAP Score (dB/m)	245 ± 30	310 ± 40	360 ± 50	88.56	< 0.001**
MRI-PDFF (%)	12.51 ± 4.26	21.63 ± 5.89	34.55 ± 6.94	102.43	< 0.001**
Mean FGF21 (pg/mL)	190 ± 40.25	260 ± 50.48	340 ± 60.12	44.28	< 0.001**
Mean ALT (U/L)	32.52 ± 12.35	46.24 ± 18.57	65.86 ± 20.44	26.18	< 0.001**
Mean AST (U/L)	30.85 ± 9.24	40.35 ± 12.76	55.67 ± 14.97	34.06	< 0.001**
Liver Volume (cm ³)	1450 ± 230	1650 ± 250	1920 ± 280	25.72	< 0.001**
Hepatic Fat Fraction (%)	8.59 ± 2.03	14.26 ± 3.55	24.67 ± 5.16	90.45	< 0.001**
Serum Albumin (g/dL)	4.13 ± 0.21	3.97 ± 0.34	3.71 ± 0.38	15.89	< 0.001**
Total Bilirubin (mg/dL)	0.82 ± 0.22	0.91 ± 0.32	1.22 ± 0.49	12.67	< 0.001**

Data are presented as Mean ± SD or n (%), as appropriate. P-values were calculated using one-way ANOVA or Chi-square tests. *P < 0.05; **P < 0.01. CAP: controlled attenuation parameter; MRI-PDFF: magnetic resonance imaging-proton density fat fraction; FGF21: fibroblast growth factor 21; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

Liver stiffness, imaging parameters, and biochemical markers according to hepatic steatosis severity

As shown in **Table 4**, liver stiffness, imaging parameters, and biochemical markers demonstrated a progressive deterioration with increasing steatosis severity. Liver stiffness, an indicator of fibrosis, increased markedly from 5.2 kPa in the mild group to 11.6 kPa in the severe group (P < 0.001). Similarly, imaging parameters showed significant worsening across severity stages. CAP increased from 245 dB/m to 360 dB/m (P = 0.001), and MRI-PDFF increased from 12.5% to 34.5% (P = 0.001), both reflecting progressively increased hepatic fat accumulation. Biochemical markers also exhibited significant changes. FGF21, a stress-responsive metabolic hormone, increased from

190 pg/mL in mild steatosis to 340 pg/mL in severe steatosis (P < 0.001), suggesting increased metabolic stress associated with disease progression. Liver enzymes were significantly elevated, with ALT increasing from 32.5 to 65.8 U/L and AST increasing from 30.8 to 55.6 U/L (both P < 0.001).

Liver volume also increased significantly from 1,450 cm³ in mild steatosis to 1,920 cm³ in severe steatosis (P < 0.001), reflecting both fat accumulation and inflammatory expansion. Hepatic fat fraction showed a more than two-fold increase, from 8.5% in the mild group to 24.6% in the severe group (P < 0.001). In contrast, serum albumin levels decreased gradually from 4.1 g/dL to 3.7 g/dL (P < 0.001), indicating a decline in hepatic synthetic function with disease progression.

Table 5. Integrated molecular signatures associated with hepatic steatosis severity and serum FGF21

Molecular Marker	Mild Steatosis	Severe Steatosis	Fold Change (Severe/Mild)	Adjusted P-value
PNPLA3 expression	1.00 ± 0.18	2.34 ± 0.41	2.34	< 0.001**
TM6SF2 expression	1.00 ± 0.15	1.89 ± 0.33	1.89	0.002*
GCKR expression	1.00 ± 0.12	1.67 ± 0.29	1.67	0.005*
FGF21 mRNA expression	1.00 ± 0.20	3.12 ± 0.54	3.12	< 0.001**
PPARα expression	1.00 ± 0.16	0.61 ± 0.11	0.61	0.011*
ATF4 expression	1.00 ± 0.17	2.48 ± 0.46	2.48	< 0.001**
Promoter DNA methylation (%)	8.4 ± 2.1	16.9 ± 4.5	2.01	< 0.001**
Free fatty acids (μmol/L)	432.6 ± 78.4	718.5 ± 122.7	1.66	< 0.001**
Acylcarnitine levels (μmol/L)	0.82 ± 0.21	1.64 ± 0.39	2.00	0.003*
Firmicutes/Bacteroidetes ratio	1.9 ± 0.5	4.3 ± 1.1	2.26	< 0.001**
Lipopolysaccharide levels (EU/mL)	0.31 ± 0.09	0.74 ± 0.18	2.39	< 0.001**

Footnotes: Fold-change values represent the ratio of expression levels in the severe steatosis group relative to the mild steatosis group (Severe/Mild). DNA methylation values are presented as mean promoter methylation percentages (%). Adjusted P-values were calculated using the Benjamini-Hochberg false discovery rate (FDR) correction for multiple testing. Data are presented as Mean ± Standard Deviation (SD) or percentage (%), as appropriate. P-values are derived from one-way analysis of variance (ANOVA), Kruskal-Wallis test, or Chi-square test, as applicable. *P < 0.05; **P < 0.01. PPARα: peroxisome proliferator-activated receptor alpha; ATF4: activating transcription factor 4; FGF21: fibroblast growth factor 21; FDR: false discovery rate.

Gut microbiome parameters by hepatic steatosis severity

Specific changes of gut microbiome parameters in the course of increasing hepatic steatosis severity are outlined in the **Table 5**. Genomic analysis demonstrated a higher frequency of key lipid metabolism-related genetic polymorphisms associated with disease severity. The prevalence of PNPLA3 increased from 25% in mild steatosis to 65% in severe steatosis (P < 0.001), TM6SF2 from 18% to 50% (P = 0.003), and GCKR from 30% to 60% (P < 0.001), all of which are implicated in lipid metabolism and hepatic fat accumulation. DNA methylation levels also increased, with a higher proportion of hypermethylated DNA observed in severe disease compared with mild disease (48% vs. 25%, P < 0.001), suggesting that epigenetic modifications may be associated with disease progression. Proteomics and metabolomic analyses indicated marked metabolic dysfunction. Proteins involved in lipid metabolism increased from 1.2 AU in mild steatosis to 3.8 AU in severe steatosis (P < 0.001). Amino acid derivatives and ketone bodies also increased significantly (from 3.5 AU to 8.2 AU and from 1.8 AU to 4.9 AU, respectively, P < 0.001), indicating altered amino acid and enhanced lipid oxidation. Likewise, free fatty acid levels showed a sharp rise from 0.4

mmol/L in mild steatosis to 1 mmol/L in severe steatosis (P < 0.001).

Gut microbiome analysis demonstrated significant dysbiosis associated with disease severity. The Firmicutes/Bacteroidetes ratio increased significantly from 1.8 in mild steatosis to 3.4 in severe steatosis (P < 0.001). In contrast, short-chain fatty acid levels decreased from 3.5 AU to 1.8 AU (P < 0.001), while the proportion of LPS-producing bacteria increased from 12% to 40% (P < 0.001), suggesting increased gut permeability and systemic inflammation. Transcriptomic analysis indicated upregulation of FGF21-regulated gene expression, increasing from 2.0 AU in mild steatosis to 5.8 AU in severe steatosis (P < 0.001), indicating its involvement in hepatic stress adaptation and metabolic regulation. These findings suggest that genetic susceptibility, epigenetic modification, metabolic dysfunction, and gut microbiota dysbiosis are interrelated in the progression of hepatic steatosis.

Integration and molecular signatures associated with FGF21

Comprehensive molecular analyses demonstrated distinct genomic, transcriptomic, proteomic, metabolomic, and microbiome-related alterations associated with hepatic steatosis

severity and circulating FGF21 levels. Genomic analysis revealed a significantly higher prevalence of NAFLD-associated polymorphisms in PNPLA3 (65% vs. 25%), TM6SF2 (50% vs. 18%), and GCKR (60% vs. 30%) in patients with severe compared with mild steatosis ($P < 0.001$), supporting the contribution of genetic susceptibility to hepatic lipid accumulation.

Transcriptomic profiling identified 312 differentially expressed genes (DEGs) between mild and severe steatosis groups ($FDR < 0.05$). Genes involved in lipid metabolism, inflammatory signaling, mitochondrial dysfunction, and oxidative stress pathways were significantly upregulated in severe steatosis. Increased expression of FGF21-associated regulatory pathways including PPAR α and ATF4 was also observed.

Proteomic analyses demonstrated increased expression of proteins associated with fatty acid oxidation, oxidative stress, and lipotoxicity. Key proteins including acyl-CoA oxidase 1 (ACOX1) and cytochrome P450 enzymes showed significantly increased expression in severe steatosis (2.5-3.2-fold increase; $P < 0.001$).

Metabolomic profiling identified significant alterations in lipid metabolism, including elevated circulating free fatty acids, acylcarnitines, and disrupted ketone body metabolism, suggesting progressive mitochondrial dysfunction with worsening steatosis severity. Pathway enrichment analyses additionally demonstrated dysregulation of fatty acid β -oxidation, bile acid metabolism, and amino acid metabolism pathways. Gut microbiome analyses demonstrated increased Firmicutes/Bacteroidetes ratio and reduced short-chain fatty acid-associated metabolic signatures in severe steatosis, supporting the role of gut dysbiosis in NAFLD progression.

Correlation analyses revealed significant associations between circulating FGF21 concentrations and multiple molecular features, including transcriptomic alterations ($r = 0.68$), proteomic markers ($r = 0.72$), and metabolomic profiles ($r = 0.75$), indicating that elevated serum FGF21 reflects widespread metabolic and inflammatory dysregulation associated with NAFLD severity. The integrated interac-

tions linking genetic, transcriptomic, proteomic, metabolomic, and microbiome alterations with circulating FGF21 levels are summarized in **Figure 1**, highlighting its central role as a systemic biomarker of metabolic stress and hepatic steatosis progression.

Multivariate logistic regression analysis

Multivariate logistic regression analysis identified several independent factors significantly associated with severe hepatic steatosis (**Table 6**). Serum FGF21 emerged as the strongest independent predictor of severe steatosis (OR = 2.10 per standard deviation increase, 95% CI: 1.75-2.53, $P < 0.001$), indicating a significant positive association between elevated circulating FGF21 concentrations and worsening hepatic fat accumulation. These findings support the role of FGF21 as a metabolic stress-responsive biomarker associated with NAFLD progression.

Markers of metabolic dysfunction and obesity were also independently associated with severe hepatic steatosis. Increased body mass index (BMI) (OR = 1.45, 95% CI: 1.23-1.71, $P < 0.001$), waist circumference (OR = 1.30, 95% CI: 1.12-1.50, $P < 0.001$), and HOMA-IR (OR = 1.60, 95% CI: 1.30-1.98, $P < 0.001$) were significant predictors, highlighting the contribution of central obesity and insulin resistance to hepatic steatosis severity.

Among liver injury markers, ALT was independently associated with severe steatosis (OR = 1.12 per 10 U/L increase, 95% CI: 1.08-1.16, $P < 0.001$). Imaging-derived parameters including liver stiffness measurement and CAP scores also demonstrated significant associations with disease severity, reflecting progressive hepatic structural alterations.

Dyslipidemia-related variables showed significant predictive value in the multivariate model. Elevated triglyceride levels were positively associated with severe hepatic steatosis (OR = 1.09, 95% CI: 1.05-1.13, $P < 0.001$), whereas higher HDL-C concentrations demonstrated a protective association (OR = 0.85, 95% CI: 0.80-0.91, $P < 0.001$).

In addition, increasing age (OR = 1.09, 95% CI: 1.06-1.12, $P < 0.001$), systemic inflammatory markers including CRP levels, and adiposity-

FGF21 as a predictor of NAFLD hepatic steatosis

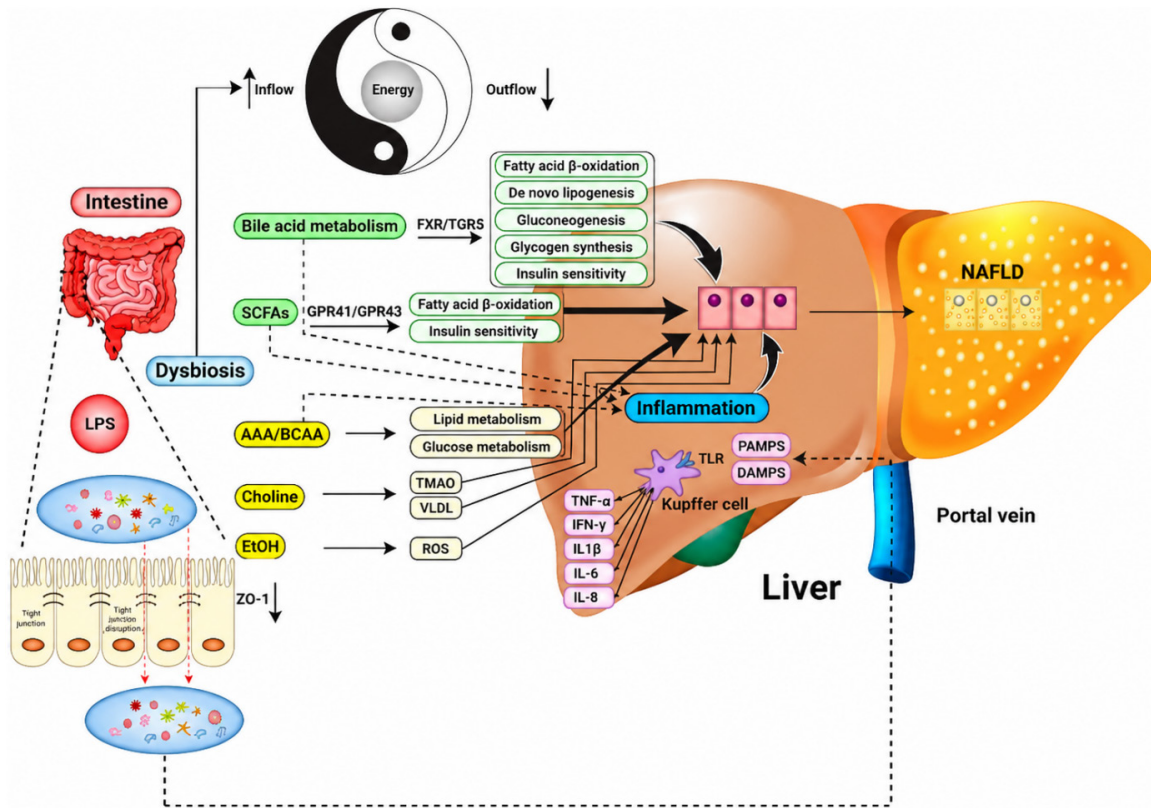


Figure 1. Molecular and metabolic alterations associated with serum FGF21 and hepatic steatosis severity.

Table 6. Multivariate logistic regression analysis for predictors of severe hepatic steatosis

Variable	Odds Ratio (OR)	95% Confidence Interval (CI)	P-value
Age (years)	1.092	1.06-1.12	< 0.001**
Serum FGF21 (per SD increase)	2.10	1.75-2.53	< 0.001**
BMI (per SD increase)	1.45	1.23-1.71	< 0.001**
HOMA-IR (per SD increase)	1.60	1.30-1.98	< 0.001**
ALT (per 10 U/L increase)	1.12	1.08-1.16	< 0.001**
Triglycerides (per SD increase)	1.09	1.05-1.13	< 0.001**
HDL-C (per SD increase)	0.85	0.80-0.91	< 0.001**
Waist Circumference (per SD increase)	1.30	1.12-1.50	< 0.001**
hs-CRP (per SD increase)	1.25	1.10-1.42	< 0.001**
IL-6 (per SD increase)	1.18	1.08-1.30	< 0.001**
Liver Stiffness (per SD increase)	1.40	1.25-1.57	< 0.001**
CAP Score (per SD increase)	1.15	1.10-1.21	< 0.001**

* $P < 0.05$; ** $P < 0.01$. FGF21: fibroblast growth factor 21; BMI: body mass index; HOMA-IR: homeostasis model assessment of insulin resistance; ALT: alanine aminotransferase; HDL-C: high-density lipoprotein cholesterol; hs-CRP: high-sensitivity C-reactive protein; IL-6: interleukin-6; CAP: controlled attenuation parameter.

related parameters contributed significantly to the risk of severe hepatic steatosis.

Overall, these findings demonstrate that severe hepatic steatosis is independently associ-

ated with metabolic dysfunction, insulin resistance, obesity, dyslipidemia, systemic inflammation, liver injury, and elevated serum FGF21 concentrations. The multivariate model further highlights the multifactorial metabolic and

Table 7. Diagnostic accuracy of serum FGF21 for predicting hepatic steatosis severity (ROC analysis)

Outcome	AUC (95% CI)	Sensitivity (%)	Specificity (%)	Cut-off (pg/mL)
Mild vs. Moderate/Severe	0.81 (0.75-0.87)	82	78	≥ 210
Moderate vs. Severe	0.88 (0.83-0.93)	85	80	≥ 290
Mild vs. Severe	0.92 (0.88-0.96)	90	85	≥ 310

AUC: area under the curve.

inflammatory mechanisms underlying NAFLD progression.

Diagnostic accuracy of serum FGF21

ROC curve analysis was performed to evaluate the diagnostic performance of serum FGF21 in distinguishing different stages of hepatic steatosis (**Table 7**). Serum FGF21 demonstrated good discriminatory ability across all comparisons, as reflected by the area under the curve (AUC), sensitivity, specificity, and optimal cutoff values. For distinguishing mild steatosis from moderate-to-severe steatosis, serum FGF21 demonstrated good diagnostic accuracy with an AUC of 0.81 (95% CI: 0.75-0.87). At an optimal cutoff value of ≥ 210 pg/mL, the sensitivity and specificity were 82% and 78%, respectively, indicating reasonable performance in identifying patients with progressive hepatic steatosis. When moderate steatosis was compared with severe steatosis, serum FGF21 showed improved diagnostic performance with an AUC of 0.88 (95% CI: 0.83-0.93). Using a cutoff value of ≥ 290 pg/mL, serum FGF21 achieved a sensitivity of 85% and a specificity of 80%, suggesting good discriminatory capacity for differentiating intermediate from advanced hepatic steatosis.

The highest diagnostic accuracy was observed when distinguishing mild steatosis from severe steatosis. In this comparison, serum FGF21 demonstrated an AUC of 0.92 (95% CI: 0.88-0.96), indicating excellent predictive performance. At a cutoff value of ≥ 310 pg/mL, the sensitivity and specificity were 90% and 85%, respectively. These findings suggest that circulating FGF21 concentrations progressively increase with worsening hepatic steatosis severity and may serve as a reliable non-invasive biomarker for disease stratification. Overall, ROC analysis demonstrated that serum FGF21 possesses strong diagnostic utility for identifying and staging hepatic steatosis severity in patients with NAFLD. The progressively increasing cutoff values observed across ste-

atosis grades further support the relationship between elevated FGF21 levels and worsening metabolic and hepatic dysfunction. However, the absence of external validation should be considered when interpreting the diagnostic performance of the model.

Weighted gene co-expression network analysis (WGCNA)

Six principal co-expression modules that correlate with severity of hepatic steatosis were identified using Weighted Gene Co-expression Network Analysis. The turquoise module (n = 186 genes) had the highest positive correlation with serum FGF21 (r = 0.74, P < 0.001) and hepatic fat fraction (FF) (r = 0.71, P < 0.001) obtained by MRI. Pathways related to lipid metabolism, mitochondrial dysfunction, fatty acid β-oxidation, inflammatory signaling, and oxidative stress were all significantly overrepresented when using functional enrichment analysis.

The blue module (n = 124 genes) had significant correlations with insulin resistance (HOMA-IR; r = 0.66, P < 0.001) and liver stiffness measurements (r = 0.63, P < 0.001). Using the hub gene analysis method, the researchers identified six central hub genes in the network as FGF21, PPARA, ATF4, CPT1A, SREBF1 and ACOX1. These genes showed a high intramodular connectivity and strong association with the transition from mild to severe hepatic steatosis.

The relationship between modules was analyzed, and the FGF21 was positively correlated with a molecular pattern of metabolic stress and inflammation, and with a pattern of lipid accumulation, suggesting that serum FGF21 is a comprehensive biomarker of disease severity.

PLS-DA demonstrated clear molecular separation among mild, moderate, and severe steatosis groups (**Figure 2**). The first two latent vari-

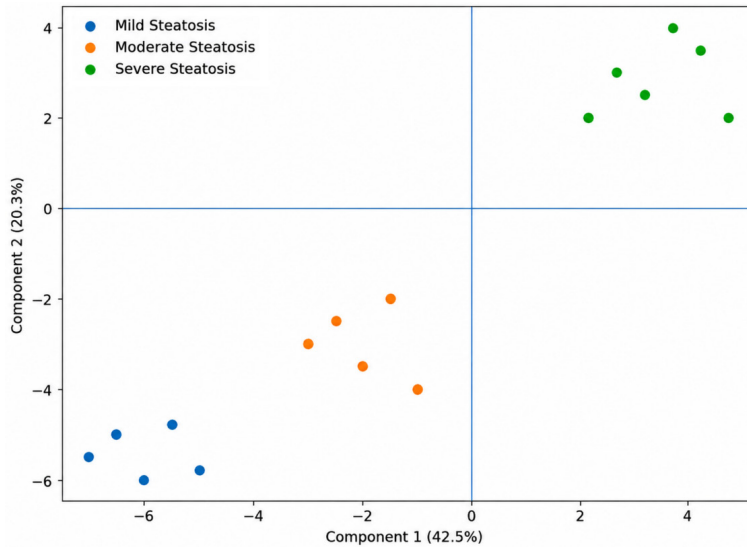


Figure 2. Partial least squares discriminant analysis (PLS-DA) score plot. This plot demonstrating molecular discrimination among mild, moderate, and severe hepatic steatosis groups. Integrated molecular variables including genomic, transcriptomic, proteomic, metabolomic, and microbiome-derived features were analyzed. Distinct clustering patterns indicate progressive molecular alterations associated with increasing steatosis severity. Component 1 explained 42.5% of the variance, whereas Component 2 explained 20.3%, accounting for a cumulative variance of 62.8%. Model performance metrics demonstrated satisfactory classification accuracy ($R^2Y = 0.81$; $Q^2 = 0.72$).

ables explained 62.8% of the total molecular variance (Component 1: 42.5%; Component 2: 20.3%). Severe steatosis samples clustered distinctly from mild steatosis samples, while moderate steatosis occupied an intermediate position, indicating progressive molecular alterations during disease progression. Model validation demonstrated strong classification performance ($R^2Y = 0.81$; $Q^2 = 0.72$; permutation $P < 0.001$), supporting the robustness of the integrated molecular profiling approach.

Discussion

The present study evaluated the association between serum FGF21, metabolic dysfunction, integrated molecular alterations, and hepatic steatosis severity in patients with NAFLD. The findings demonstrated that increasing steatosis severity was significantly associated with obesity, insulin resistance, dyslipidemia, systemic inflammation, oxidative stress, liver injury, and elevated circulating FGF21 concentrations. In addition, integrated molecular analyses revealed progressive alterations in genomic, transcriptomic, metabolomic, pro-

teomic, and microbiome-related signatures across disease severity groups. Collectively, these findings support the multifactorial metabolic and inflammatory basis of NAFLD progression and highlight the potential role of serum FGF21 as a non-invasive biomarker of hepatic steatosis severity.

In the present study, BMI and waist circumference progressively increased with worsening hepatic steatosis severity. Patients with severe steatosis demonstrated significantly higher BMI and greater central adiposity compared with those with mild disease. These findings are consistent with previous studies demonstrating that obesity and metabolic dysfunction are major contributors to hepatic fat accumulation and NAFLD progression [1, 2, 12]. Central obesity is strongly associated with in-

creased free fatty acid flux to the liver, insulin resistance, and chronic low-grade inflammation, all of which promote hepatic lipid deposition and progression toward steatohepatitis and fibrosis. Insulin resistance also showed a strong association with hepatic steatosis severity in the current study. HOMA-IR values increased significantly across steatosis groups, indicating worsening metabolic dysfunction with disease progression. These findings are consistent with previous investigations identifying insulin resistance as a central mechanism in NAFLD pathogenesis [12, 13]. Excessive insulin resistance promotes hepatic de novo lipogenesis, impaired fatty acid oxidation, and triglyceride accumulation within hepatocytes, thereby contributing to steatosis progression and hepatic injury. Lifestyle-related factors including reduced physical activity and increased caloric intake were also significantly associated with severe hepatic steatosis. Patients with advanced disease demonstrated lower physical activity levels and higher daily caloric intake, particularly from saturated fats. These observations are supported by evidence showing that diet, exercise, and sedentary

behavior influence hepatic steatosis and insulin resistance in NAFLD [24]. Increased saturated fat intake may further exacerbate oxidative stress, mitochondrial dysfunction, and inflammatory signaling pathways involved in NAFLD progression.

The present study additionally demonstrated significant elevations in liver enzymes, inflammatory markers, and oxidative stress parameters with increasing disease severity. Serum ALT, AST, and GGT levels progressively increased across steatosis groups, reflecting worsening hepatocellular injury. Similarly, inflammatory markers including hs-CRP, IL-6, and TNF- α were significantly elevated in patients with severe steatosis, supporting the contribution of systemic inflammation to NAFLD progression. Oxidative stress markers such as malondialdehyde (MDA) were also increased, whereas total antioxidant capacity declined with worsening steatosis severity. These findings are consistent with current evidence indicating that oxidative stress, lipotoxicity, and inflammatory activation play central roles in the transition from simple steatosis to steatohepatitis and fibrosis [12, 13]. Imaging parameters including liver stiffness measurement, CAP, and MRI-PDFF also demonstrated progressive increases with worsening hepatic steatosis. These findings further validate the relationship between metabolic dysfunction and structural hepatic alterations. MRI-PDFF and elastography-based measurements have previously been shown to correlate closely with hepatic fat content and fibrosis severity in NAFLD patients [4, 5].

One of the principal findings of the current study was the significant elevation of serum FGF21 concentrations with increasing hepatic steatosis severity. Circulating FGF21 levels progressively increased from mild to severe steatosis, supporting previous evidence that FGF21 functions as a metabolic stress-responsive hepatokine [6-11, 20, 21]. Elevated FGF21 concentrations likely reflect compensatory responses to hepatic lipid accumulation and metabolic stress, and insulin resistance. Experimental studies have shown that FGF21 is induced under conditions of metabolic stress through activation of pathways involving peroxisome proliferator-activated receptor α (PPAR α) and activating transcription factor 4 (ATF4), both of which regulate lipid metabolism

and adaptive metabolic responses [18, 19]. Importantly, multivariate logistic regression analysis demonstrated that serum FGF21 was the strongest independent predictor of severe hepatic steatosis after adjustment for confounding variables including age, BMI, and HOMA-IR. Elevated serum FGF21 concentrations were independently associated with approximately two-fold higher odds of severe hepatic steatosis. These findings agree with previous clinical studies reporting strong associations between circulating FGF21 levels and advanced NAFLD, steatohepatitis, and fibrosis severity [9, 10, 20-22]. The independent predictive value of FGF21 observed in the present study suggests that FGF21 may represent a clinically useful biomarker reflecting integrated metabolic and inflammatory stress associated with NAFLD progression.

The integrated molecular analyses performed in this study further provided insight into the biological mechanisms associated with worsening hepatic steatosis. Increased expression of NAFLD-associated genes including PNPLA3, TM6SF2 and GCKR, was observed in patients with severe disease. These genetic variants have previously been implicated in hepatic lipid accumulation, impaired lipid export, and increased susceptibility to fibrosis progression [14, 15]. Furthermore, transcriptomic analyses demonstrated upregulation of FGF21-related signaling pathways and metabolic stress-associated genes in advanced steatosis. Epigenetic analyses additionally revealed increased promoter methylation of genes involved in lipid metabolism and inflammatory signaling in severe disease groups. These findings support growing evidence that epigenetic modifications contribute to NAFLD pathogenesis by regulating hepatic metabolic pathways and inflammatory responses [15, 25]. Proteomic and metabolomic analyses also demonstrated progressive alterations in lipid metabolism proteins, free fatty acids, acylcarnitines, amino acid derivatives, and ketone body metabolism with worsening steatosis severity, suggesting substantial mitochondrial and metabolic dysfunction. Gut microbiome analysis revealed significant dysbiosis in severe hepatic steatosis, characterized by increased Firmicutes/Bacteroidetes ratio and reduced SCFA production. Previous studies have demonstrated that gut microbiota alterations contribute to NAFLD pro-

gression through changes in microbial metabolites, intestinal barrier function, and inflammatory signaling [16, 17]. Reduced SCFA production may further impair anti-inflammatory and metabolic regulatory pathways involved in hepatic protection.

ROC curve analysis demonstrated good-to-excellent diagnostic performance of serum FGF21 for differentiating hepatic steatosis severity stages. Serum FGF21 achieved the highest diagnostic accuracy when distinguishing mild from severe steatosis, with high sensitivity and specificity. These findings are consistent with previous studies showing that circulating FGF21 is associated with NAFLD and may identify higher-grade hepatic steatosis [9, 20-22]. The progressively increasing cutoff values observed across steatosis grades further support the close association between FGF21 concentrations and worsening hepatic metabolic dysfunction. The present study has several important clinical implications. First, serum FGF21 may serve as a non-invasive biomarker for identifying patients at increased risk of progressive hepatic steatosis. Second, the integration of molecular and metabolic analyses highlights the complex pathophysiological mechanisms underlying NAFLD progression. Finally, these findings support ongoing interest in FGF21-targeted therapeutic approaches for metabolic liver disease.

In conclusion, the present study demonstrated that serum FGF21 is strongly associated with hepatic steatosis severity and independently predicts severe NAFLD. Increasing disease severity was accompanied by progressive metabolic dysfunction, systemic inflammation, oxidative stress, imaging abnormalities, and integrated molecular alterations. These findings support the utility of serum FGF21 as a promising non-invasive biomarker for risk stratification and disease assessment in patients with NAFLD.

Significance of the study

The current work underscores the importance of serum FGF21 as a robust marker of the severity of hepatic steatosis in NAFLD, pointing to its application in non-invasive diagnosis and disease staging. The results emphasize the crosstalk between metabolic, inflammatory, and structural alterations in NAFLD and the associated markers including BMI, HOMA-IR,

LS, and dyslipidemia. Therefore, this study offers a valuable guide for assessing the risk of NAFLD development and stratifying patient risk, which may facilitate early targeted prevention and treatment strategies.

Limitations of the study

Despite its strengths, this study has several limitations. The retrospective study design limits the ability to establish causal relationships between serum FGF21 and NAFLD progression. The relatively modest sample size and absence of external validation may also limit the generalizability of the findings. In addition, although integrated molecular analyses were performed, the depth of omics characterization remained limited compared with large-scale systems biology studies. Potential overfitting of predictive models should also be considered when interpreting the ROC analyses.

Furthermore, the retrospective design prevents causal inference between biomarkers and disease progression. The study population consisted of relatively young adults, which may introduce selection bias. Further, although FGF21 had emerged as the most significant biomarker, we have not thoroughly investigated the possible interactions with other metabolic diseases. Finally, data were collected using serum biomarkers and imaging techniques; while non-invasive, they do not provide the histological detail of liver biopsy.

Conclusion

This study demonstrates that serum FGF21 and other clinical indices may serve as diagnostic tools for severe hepatic steatosis in NAFLD. The results imply that FGF21 may be employed in practice as a biomarker for monitoring disease status and its development. Nevertheless, more longitudinal assessments of these biomarkers as a treatment target or an early diagnostic test for NAFLD, as well as the combined use of FGF21 with other biomarkers, are needed for further confirmation. Thus, this study adds value to the biomarker field and provides insights into NAFLD behavior with disease severity.

Disclosure of conflict of interest

None.

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References

- [1] Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L and Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 2016; 64: 73-84.
- [2] Targher G, Tilg H and Byrne CD. Non-alcoholic fatty liver disease: a multisystem disease requiring a multidisciplinary and holistic approach. *Lancet Gastroenterol Hepatol* 2021; 6: 578-588.
- [3] Cao Y, Deng Y, Wang J, Zhao H, Zhang J and Xie W. The association between NAFLD and risk of chronic kidney disease: a cross-sectional study. *Ther Adv Chronic Dis* 2021; 12: 20406223211048649.
- [4] Castera L, Friedrich-Rust M and Loomba R. Noninvasive assessment of liver disease in patients with nonalcoholic fatty liver disease. *Gastroenterology* 2019; 156: 1264-1281, e4.
- [5] Gu Q, Cen L, Lai J, Zhang Z, Pan J, Zhao F, Yu C, Li Y, Chen C, Chen W and Shen Z. A meta-analysis on the diagnostic performance of magnetic resonance imaging and transient elastography in nonalcoholic fatty liver disease. *Eur J Clin Invest* 2021; 51: e13446.
- [6] Fisher FM and Maratos-Flier E. Understanding the physiology of FGF21. *Annu Rev Physiol* 2016; 78: 223-241.
- [7] Badakhshi Y and Jin T. Current understanding and controversies on the clinical implications of fibroblast growth factor 21. *Crit Rev Clin Lab Sci* 2021; 58: 311-328.
- [8] Su X, Kong Y and Peng D. Fibroblast growth factor 21 in lipid metabolism and non-alcoholic fatty liver disease. *Clin Chim Acta* 2019; 498: 30-37.
- [9] Yilmaz Y, Eren F, Yonal O, Kurt R, Aktas B, Celikel CA, Ozdogan O, Imeryuz N, Kalayci C and Avsar E. Increased serum FGF21 levels in patients with nonalcoholic fatty liver disease. *Eur J Clin Invest* 2010; 40: 887-892.
- [10] Li H, Fang Q, Gao F, Fan J, Zhou J, Wang X, Zhang H, Pan X, Bao Y, Xiang K, Xu A and Jia W. Fibroblast growth factor 21 levels are increased in nonalcoholic fatty liver disease patients and are correlated with hepatic triglyceride. *J Hepatol* 2010; 53: 934-940.
- [11] Falamarzi K, Malekpour M, Tafti MF, Azarpira N, Behboodi M and Zarei M. The role of FGF21 and its analogs on liver associated diseases. *Front Med (Lausanne)* 2022; 9: 967375.
- [12] Parthasarathy G, Revelo X and Malhi H. Pathogenesis of nonalcoholic steatohepatitis: an overview. *Hepatol Commun* 2020; 4: 478-492.
- [13] Hughey CC, Puchalska P and Crawford PA. Integrating the contributions of mitochondrial oxidative metabolism to lipotoxicity and inflammation in NAFLD pathogenesis. *Biochim Bio-phys Acta Mol Cell Biol Lipids* 2022; 1867: 159209.
- [14] Eslam M and George J. Genetic contributions to NAFLD: leveraging shared genetics to uncover systems biology. *Nat Rev Gastroenterol Hepatol* 2020; 17: 40-52.
- [15] Eslam M, Valenti L and Romeo S. Genetics and epigenetics of NAFLD and NASH: clinical impact. *J Hepatol* 2018; 68: 268-279.
- [16] Hrnčir T, Hrnčířová L, Kverka M, Hromádka R, Machová V, Trčková E, Kostovčíková K, Kralícková P, Krejsek J and Tlaskalová-Hogenová H. Gut microbiota and NAFLD: pathogenic mechanisms, microbiota signatures and therapeutic interventions. *Microorganisms* 2021; 9: 957.
- [17] Kasubuchi M, Hasegawa S, Hiramatsu T, Ichimura A and Kimura I. Dietary gut microbial metabolites, short-chain fatty acids and host metabolic regulation. *Nutrients* 2015; 7: 2839-2849.
- [18] Inagaki T, Dutchak P, Zhao G, Ding X, Gautron L, Parameswara V, Li Y, Goetz R, Mohammadi M, Esser V, Elmquist JK, Gerard RD, Burgess SC, Hammer RE, Mangelsdorf DJ and Kliewer SA. Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. *Cell Metab* 2007; 5: 415-425.
- [19] De Sousa-Coelho AL, Marrero PF and Haro D. Activating transcription factor 4-dependent induction of FGF21 during amino acid deprivation. *Biochem J* 2012; 443: 165-171.
- [20] Li H, Dong K, Fang Q, Hou X, Zhou M, Bao Y, Xiang K, Xu A and Jia W. High serum level of fibroblast growth factor 21 is an independent predictor of non-alcoholic fatty liver disease: a 3-year prospective study in China. *J Hepatol* 2013; 58: 557-563.
- [21] Xiao F, Shi X, Huang P, Zeng X, Wang L, Zeng J, Liu C, Yan B, Song H, Xu Y, Han L, Zhao Q, Lin M and Li X. Dose-response relationship between serum fibroblast growth factor 21 and liver fat content in non-alcoholic fatty liver disease. *Diabetes Metab* 2021; 47: 101221.
- [22] Hua MC, Huang JL, Hu CC, Yao TC and Lai MW. Including fibroblast growth factor-21 in combined biomarker panels improves predictions of liver steatosis severity in children. *Front Pediatr* 2019; 7: 420.

- [23] Chang Y, Gao XQ, Shen N, He J, Fan X, Chen K, Lin XH, Li HM, Tian FS and Li H. A targeted metabolomic profiling of plasma acylcarnitines in nonalcoholic fatty liver disease. *Eur Rev Med Pharmacol Sci* 2020; 24: 7433-7441.
- [24] Mahady SE and George J. Exercise and diet in the management of nonalcoholic fatty liver disease. *Metabolism* 2016; 65: 1172-1182.
- [25] Hyun J and Jung Y. DNA methylation in nonalcoholic fatty liver disease. *Int J Mol Sci* 2020; 21: 8138.