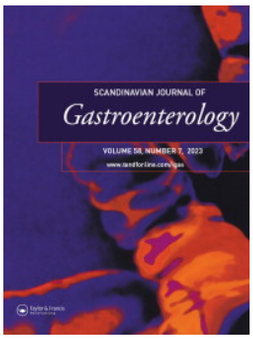

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ORIGINAL ARTICLE

Mass spectrometry-based untargeted metabolomics study of non-obese individuals with non-alcoholic fatty liver disease

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ABSTRACT

Objectives: Non-alcoholic fatty liver disease (NAFLD) is a disease characterized by the accumulation of excessive fat in the liver, which can lead to fibrosis and has an increasing prevalence. NAFLD requires non-invasive diagnostic biomarkers. While typically observed in overweight individuals, it can also occur in non-obese/non-overweight individuals. Comparative studies on non-obese NAFLD patients are scarce. This study aimed to conduct a using liquid chromatography-high resolution mass spectrometry (LC-MS/MS)-based metabolic profiling of non-obese NAFLD patients and healthy controls.

Materials and methods: The patient group consisted of 27 individuals with NAFLD, while the healthy control group included 39 individuals. Both groups were between 18 and 40 years old, had a BMI of less than 25 and had alcohol consumption less than 20 g/week for men and 10 g/week for women. Serum samples were collected and analyzed using LC-MS/MS. The data were analyzed using the TidyMass and MetaboAnalyst.

Results: The LC-MS/MS analyses detected significant changes in D-amino acid metabolism, vitamin B6 metabolism, apoptosis, mTOR signaling pathway, lysine degradation, and phenylalanine metabolism pathways in non-obese NAFLD patients. Significant changes were also observed in the metabolites D-pantothenic acid, hypoxanthine, citric acid, citramalic acid, L-phenylalanine, glutamine, and histamine-trifluoromethyl-toluidide, β-hydroxymyristic acid, DL-Lactic acid, and 3-methyl-2-oxopentanoic. Overall, the study provides valuable insights into the metabolic changes associated with non-obese NAFLD patients and can contribute to the development of non-invasive diagnostic biomarkers for NAFLD.

Conclusions: This study sheds light on the metabolic changes in non-obese NAFLD patients. Further research is needed to better understand the metabolic changes associated with NAFLD and to develop effective treatment options.

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Metabolomics; non-obese; non-alcoholic fatty liver disease; nafld; metabolites

Introduction

Nonalcoholic fatty liver disease (NAFLD) is currently the most common cause of chronic liver disease globally, affecting about a quarter of the adult population. NAFLD is a metabolic disorder that ranges from simple hepatic steatosis to inflammation, fibrosis, and cirrhosis [1,2]. NAFLD is associated with obesity, insulin resistance, and other metabolic disturbances. The development of nonalcoholic steatohepatitis (NASH) involves alterations in hepatic lipid metabolism, oxidative stress, mitochondrial dysfunction, DNA damage, and release of various cytokines [1,3].

NAFLD progresses from simple hepatic steatosis to NASH, which is characterized by inflammation and hepatocyte injury, potentially leading to cirrhosis, liver failure, and hepatocellular carcinoma. Liver biopsy is the gold standard for NASH diagnosis, but it is impractical for clinical diagnostics or disease monitoring due to its invasiveness and interobserver variability [4]. Thus, numerous noninvasive tests have been developed to identify patients with NASH or at a higher risk of developing it. However, there is no single biomarker or cluster of metabolites in the plasma that has been conclusively linked to the progression of the disease [5].

Metabolomics is a promising approach for identifying biomarkers to differentiate NAFLD patients and different presentations of NAFLD [6]. Evaluating the plasma metabolomics profile of biopsy-proven NAFLD patients to find specific biomarkers is essential to differentiate them from healthy individuals and between different forms of NAFLD. Comparing

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the metabolomics profiles of different types of cirrhosis can also help identify any differences in the metabolomic profile of these diseases [7].

NAFLD is a common liver disorder that is often studied in association with obese individuals. However, NAFLD can also occur in non-obese individuals [8–10]. The aim of this study is to conduct metabolic profiling in non-obese NAFLD patients. Besides, understanding the metabolic profile of non-obese NAFLD patients can provide important insights into the pathophysiology of the disease and potentially lead to the development of targeted therapies.

Material and methods

The study included patients with non-alcoholic fatty liver disease who presented to the Gastroenterology and Hepatology Department of Bezmialem Vakif University Hospital. The research was conducted in accordance with the principles of the 1964 Helsinki Declaration. The clinical research ethics committee of Bezmialem Vakif University approved the study (No: 2022/46). The patient group consisted of 27 individuals without any liver disease other than Non-Alcoholic Fatty Liver Disease. Additionally, 39 individuals without any liver disease, including non-alcoholic fatty liver disease, were included as the healthy control group. Both groups consisted of individuals between 18 and 40 years old, with a body mass index of less than 25, who did not use any medication or dietary supplements, and had alcohol consumption less than 20 g/week for men and 10 g/week for women.

The blood samples collected in tubes without separator gel were centrifuged at 3500 RPM for 10 min and stored at −86 °C. Five hundred microliters of the thawed serum samples were transferred to a different Eppendorf tube, and 1 mL of methanol was added. The mixture was centrifuged at 10,000 g for 1 h, and the upper layer of the obtained samples was transferred to HPLC vials. Mass spectrometry analyses were performed using liquid chromatography-high resolution mass spectrometry (LC-HRMS) on a Thermo Q Exactive instrument. Isocratic flow with a mobile phase of methanol and a Fortis C18, 3 µm particle size, 150 × 2 mm column was used for chromatographic separation. Positive/negative ESI full scan was performed in mass spectrometry analyses. The biochemical tests were performed using commercial medical diagnostic kits on the Siemens Atellica® CH 930 analyzer.

The raw data from mass spectrometry were converted into mzXML and mgf formats using the open source ProteoWizard software [11]. The converted data were analyzed using TidyMass and MetaboAnalyst R packages for metabolite analysis [12,13]. The public mass spectrum databases HMDB (The Human Metabolome Database), MassBank, and MoNA (MassBank of North America) were used for metabolite annotation. The public pathway database KEGG (Kyoto Encyclopedia of Genes and Genomes) was used for pathway analysis. Statistical analyses were performed using the R-Project (v.4.2.2) open source coding language [14].

Results

In the results of this study, alterations in the metabolite profile of non-obese NAFLD patients, a niche population, compared to healthy control individuals have been demonstrated. Table 1 provided contains descriptive statistics for various variables in a study involving a control group ($N = 39$) and a patient group ($N = 27$). For BMI, the mean is 21.0 for the control group and 22.7 for the patient group, with a total mean of 21.7. The minimum BMI is 17.0 for both groups, and the maximum BMI is 25.0 for both groups. The median BMI is 20.8 for the control group, 22.8 for the patient group, and 21.4 for the total sample.

After processing the raw data obtained by mass spectrometry, metabolite annotation was performed, and the fold change analysis results of the metabolites showing significant differences between the groups were visualized. In individuals with NAFLD who were not obese, it was determined that the metabolites D-pantothenic acid, hypoxanthine, citric acid, citramalic acid, L-phenylalanine, glutamine, tramadol, 1,4-butynediol, DL-pyroglutamic acid, dehydroisoandrosterone sulfate (DHEA-S), 5-androsten-3-β,17-β-diol-3-sulfate, glyceric acid, D-ribose, and 5-αpregnan-3-α-,17-diol-20-one 3-sulfate were significantly higher compared to the non-obese healthy control group. On the other hand, it was found that the metabolites β-hydroxymyristic acid, histamine-trifluoromethyl-toluidide, DL-Lactic acid, and 3-methyl-2-oxopentanoic acid were significantly lower (Figure 1 and Table 2).

Metabolites with significant differences between the groups were subjected to pathway analysis, which revealed their association with various pathways including D-amino acid metabolism, aminoacyl-tRNA biosynthesis, arginine biosynthesis, ABC transporters, alanine, aspartate and glutamate metabolism, vitamin B6 metabolism, apoptosis, mTOR signaling pathway, lysine degradation, and phenylalanine metabolism (Figure 2).

Conclusion

NAFLD is a growing health concern worldwide, and the lack of non-invasive diagnostic biomarkers has hampered the effective management of this disease. This study aimed to shed light on the metabolic changes associated with non-obese/non-overweight NAFLD patients by conducting mass spectrometry-based metabolic profiling. The results of this study provide valuable insights into the metabolic changes in non-obese/non-overweight NAFLD patients and highlight the importance of developing non-invasive diagnostic biomarkers for this disease. The findings of this study could help pave the way for the development of effective treatment options for NAFLD.

In non-obese NAFLD patients, the metabolites Histamine-trifluoromethyl-toluidide, β-hydroxymyristic acid, DL-Lactic acid, and 3-methyl-2-oxopentanoic acid are down-regulated. Histamine-trifluoromethyl-toluidide acts as an agonist for Histamine H1/H2 receptors. Recent studies have proposed two potential mechanisms associated with histamine receptors that could mitigate liver damage during the early stages of NAFLD progression [15]. These mechanisms

Table 1. Demographic and clinical characteristics of participants.

		Control ($N=39$)	Patient ($N=27$)	Total ($N=66$)	p-value
Age	Mean (SD)	22.4 (0.8)	24.8 (5.2)	23.4 (3.5)	.006[a]
	Min–Max	20.0–24.0	20.0–39.0	20.0–39.0	
	Median (IQR)	22.0 (22.0–23.0)	23.0 (22.0–25.0)	22.5 (22.0–23.0)	.264[b]
Weight (kg)	Mean (SD)	60.1 (9.9)	67.2 (10.3)	63.0 (10.6)	.007[a]
	Min–Max	47.9–86.3	47.7–93.0	47.7–93.0	
	Median (IQR)	56.6 (53.1–64.8)	68.9 (62.0–72.2)	62.8 (54.1–70.1)	.005[b]
Height (cm)	Mean (SD)	168.6 (9.1)	171.7 (9.1)	169.9 (9.1)	.176[a]
	Min–Max	155.0–200.0	156.0–195.0	155.0–200.0	
	Median (IQR)	167.0 (163.0–172.0)	170.0 (165.5–179.5)	168.0 (164.2–176.8)	.120[b]
BMI	Mean (SD)	21.0 (2.1)	22.7 (2.0)	21.7 (2.2)	.002[a]
	Min–Max	17.0–25.0	18.2–24.9	17.0–25.0	
	Median (IQR)	20.8 (19.4–22.5)	22.8 (21.4–24.6)	21.4 (20.0–23.8)	.002[b]
Abdominal fat (kg)	Mean (SD)	5.7 (2.1)	7.4 (2.8)	6.5 (2.5)	.022[a]
	Min–Max	3.2–10.6	3.3–13.2	3.2–13.2	
	Median (IQR)	4.9 (4.0–7.6)	6.1 (5.4–9.6)	5.7 (4.3–8.4)	.023[b]
Glucose (mg/dL)	Mean (SD)	87.9 (6.9)	92.8 (7.8)	89.9 (7.6)	.010[a]
	Min–Max	64.0–102.0	84.0–117.0	64.0–117.0	
	Median (IQR)	87.0 (86.0–91.5)	91.0 (87.5–96.5)	88.5 (87.0–93.5)	.023[b]
Insulin (mIU/L)	Mean (SD)	6.7 (2.6)	6.7 (2.6)	6.7 (2.6)	.960[a]
	Min–Max	2.4–13.5	1.6–14.4	1.6–14.4	
	Median (IQR)	6.3 (5.0–8.0)	5.8 (4.9–7.8)	6.1 (4.9–7.9)	.932[b]
Homa-IR	Mean (SD)	1.5 (0.6)	1.5 (0.6)	1.5 (0.6)	.622[a]
	Min–Max	0.5–3.4	0.3–3.1	0.3–3.4	
	Median (IQR)	1.3 (1.1–1.8)	1.4 (1.2–1.8)	1.3 (1.1–1.8)	.620[b]
Cholesterol (mg/dL)	Mean (SD)	174.1 (31.3)	165.2 (32.6)	170.5 (31.9)	.269[a]
	Min–Max	124.0–235.0	118.0–248.0	118.0–248.0	
	Median (IQR)	165.0 (151.5–195.0)	150.0 (144.5–179.5)	164.5 (148.0–194.0)	.167[b]
Triglyceride (mg/dL)	Mean (SD)	65.9 (22.5)	78.1 (39.6)	70.9 (31.0)	.117[a]
	Min–Max	37.0–145.0	34.0–221.0	34.0–221.0	
	Median (IQR)	61.0 (49.5–81.5)	67.0 (53.5–86.5)	62.5 (51.0–81.8)	.225[b]
HDL (mg/dL)	Mean (SD)	64.6 (12.4)	56.5 (12.9)	61.3 (13.1)	.013[a]
	Min–Max	44.9–87.9	39.0–86.8	39.0–87.9	
	Median (IQR)	64.1 (54.2–75.0)	55.6 (46.0–60.5)	59.2 (50.9–71.8)	.012[b]
LDL (mg/dL)	Mean (SD)	102.4 (28.3)	101.0 (29.5)	101.9 (28.5)	.845[a]
	Min–Max	55.7–149.4	52.3–179.7	52.3–179.7	
	Median (IQR)	102.9 (76.2–121.9)	92.2 (81.9–114.7)	100.6 (78.7–119.9)	.676[b]
ALT (IU/L)	Mean (SD)	16.7 (13.3)	27.2 (24.7)	21.0 (19.3)	.029[a]
	Min–Max	6.0–76.0	9.0–133.0	6.0–133.0	
	Median (IQR)	12.0 (10.0–15.5)	19.0 (13.5–32.5)	14.0 (11.0–23.8)	.005[b]
AST (IU/L)	Mean (SD)	18.0 (7.9)	20.3 (8.6)	19.0 (8.2)	.272[a]
	Min–Max	8.0–51.0	8.0–46.0	8.0–51.0	
	Median (IQR)	16.0 (14.0–19.0)	19.0 (14.0–24.5)	16.0 (14.0–21.8)	.218[b]
ALP (IU/L)	Mean (SD)	61.7 (20.2)	62.6 (16.9)	62.1 (18.8)	.848[a]
	Min–Max	39.0–149.0	39.0–99.0	39.0–149.0	
	Median (IQR)	56.0 (51.0–70.5)	58.0 (49.5–75.0)	57.0 (49.5–71.8)	.518[b]
GGT (IU/L)	Mean (SD)	12.8 (8.7)	21.5 (15.0)	16.4 (12.4)	.004[a]
	Min–Max	7.0–57.0	7.0–78.0	7.0–78.0	
	Median (IQR)	10.0 (9.0–13.5)	19.0 (11.0–23.0)	12.0 (10.0–18.8)	.001[b]
Total bilirubin (mg/dL)	Mean (SD)	0.7 (0.3)	1.0 (0.5)	0.8 (0.4)	.012[a]
	Min–Max	0.3–1.7	0.2–2.2	0.2–2.2	
	Median (IQR)	0.7 (0.5–0.8)	0.9 (0.6–1.3)	0.7 (0.5–1.0)	.028[b]
Vitamin D (ng/mL)	Mean (SD)	25.4 (11.9)	24.0 (10.0)	24.8 (11.1)	.643[a]
	Min–Max	4.5–60.1	8.7–47.8	4.5–60.1	
	Median (IQR)	22.3 (19.2–27.7)	22.0 (16.6–27.9)	22.3 (18.1–27.8)	.600[b]
Vitamin B12 (pg/mL)	Mean (SD)	356.6 (151.7)	430.3 (388.4)	387.1 (274.9)	.298[a]
	Min–Max	178.0–843.0	159.0–1905.0	159.0–1905.0	
	Median (IQR)	329.0 (263.0–435.0)	355.0 (265.0–398.8)	331.0 (263.5–415.5)	.933[b]
Copper (µg/dL)	Mean (SD)	94.8 (18.2)	102.7 (19.6)	98.0 (19.1)	.098[a]
	Min–Max	63.9–138.5	74.2–168.4	63.9–168.4	
	Median (IQR)	92.8 (82.9–104.8)	98.9 (92.1–111.2)	93.9 (87.6–107.9)	.088[b]
Zinc (µg/dL)	Mean (SD)	83.2 (10.7)	91.1 (12.5)	86.4 (12.0)	.007[a]
	Min–Max	50.0–100.0	66.0–118.0	50.0–118.0	
	Median (IQR)	86.0 (76.0–89.5)	92.0 (86.0–97.5)	87.0 (80.0–94.8)	.009[b]

[a]Represents the p-value of the student's t-test.
[b]Represents the p-value of the Mann-Whitney U test.

involve the regulation of cholesterol and bile acid metabolism. Histamine receptors exert a short-term inhibitory effect on cholesterol absorption when exposed to a high cholesterol diet. Additionally, in the context of a high cholestasis diet, histamine signaling leads to decreased serum bile acid levels and increased fecal bile acid levels. Consequently, histamine receptor signaling has been observed to significantly alleviate liver damage in both the initial and progressive phases of NAFLD [15,16].

Furthermore, histamine may also modulate metabolic and inflammatory processes in other organs targeted by the metabolic syndrome. In contrast, the levels of L-Histidine, the

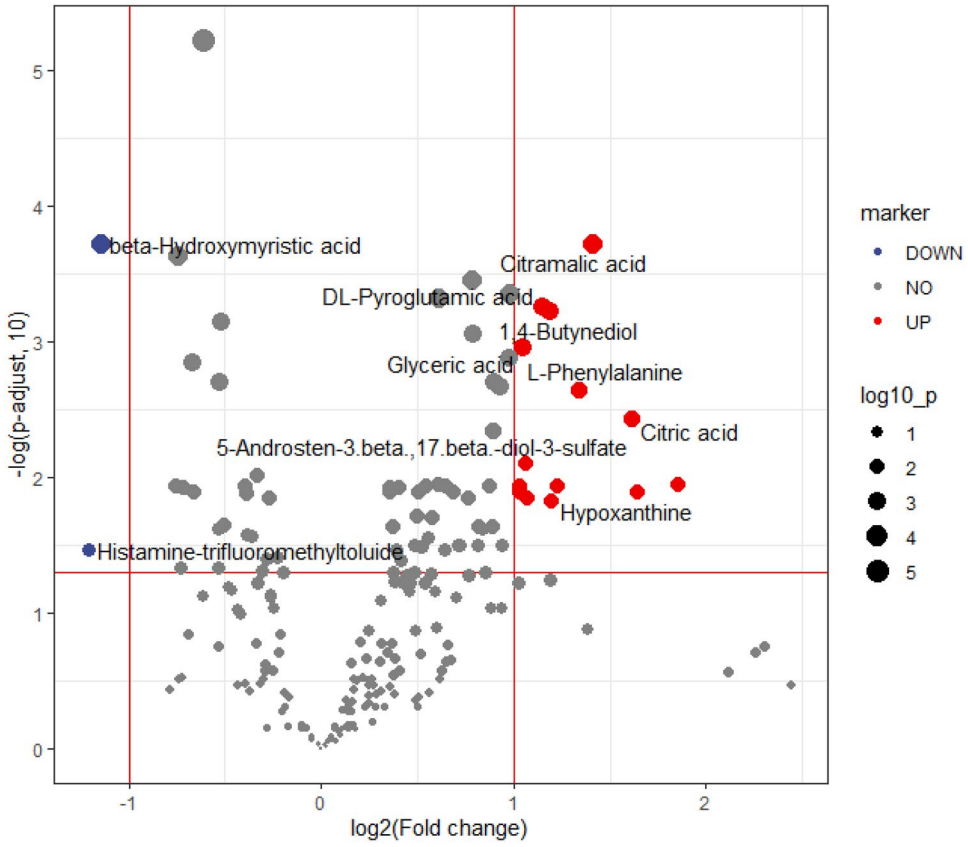

Figure 1. Visualization of metabolites exhibiting significant Fold-Change differences between groups (upregulated metabolites in the patient group shown in red circles, and downregulated metabolites shown in blue circles relative to the control group). the x-axis represents log2(fold change), and the y-axis represents – log(*p*-adjust, 10) values.

precursor for histamine synthesis in metabolism, were observed to be markedly elevated in the patient group. This finding implies that the downregulation of receptors may occur as a metabolic response. Therefore, H1/H2 receptor antagonists hold potential as therapeutic options for managing metabolic syndrome.

In non-obese NAFLD patients, there appears to be a decrease is observed in lactic acid levels. This observation may be explained by the utilization of lactic acid as a substrate for hepatic gluconeogenesis, a process involved in the production of glucose by the liver.

2-Hydroxymyristic acid is a compound similar to myristic acid that functions as an inhibitor of myristoyl-CoA:protein N-myristoyltransferase, an enzyme involved in the process of protein N-myristoylation. Myristoylation is an example of a protein-lipid modification that serves essential functions in cellular signaling, protein-protein interactions, and the localization of proteins within endomembrane and plasma membrane systems [17]. When treated with 2-hydroxymyristic acid, myristoylation is hindered, leading to alterations in the stability of specific proteins [18]. The perturbation of this protein modification pathway in non-obese NAFLD patients may have implications for the overall cellular function and homeostasis. Moreover, low levels of 3-Methyl-2-oxopentanoic acid indicate an incomplete breakdown of branched-chain amino acids, suggesting potential disturbances in amino acid metabolism.

These alterations in amino acid metabolism could impact various cellular processes and metabolic pathways, potentially contribute to the pathogenesis of NAFLD in non-obese individuals. These metabolic disruptions may be attributed to the occurrence of steatosis, a condition characterized by the accumulation of fat in the liver. Further investigation is warranted to gain a comprehensive understanding of the underlying mechanisms linking these metabolic changes to the development and progression of NAFLD in non-obese individuals.

Lee et al. have reported that in non-obese individuals with NAFLD, the levels of short-chain fatty acids (SCFAs) acetate and propionate increase with the progression of fibrosis. Conversely, in obese individuals with NAFLD, no significant association between SCFA levels and fibrosis severity was observed. Moreover, Lee et al. observed higher fecal levels of several conjugated and unconjugated bile acids in non-obese individuals with fibrosis, while in obese individuals, the levels of total conjugated bile acids were negatively correlated with the severity of fibrosis [19].

Aggarwal et al. investigated the effects of the palmitate metabolite on NAFLD patients. They identified changes in the amino acid pathway and observed an increase in the phenylalanine metabolite. In our study, we also identified changes in the amino acid pathway and phenylalanine metabolite. While Aggarwal et al. conducted their research on cell cultures and obese individuals, our study focused on non-obese

Table 2. Adjusted *p*-value, *p*-value, and fold-change values for annotated metabolites from the analysis results.

Fold-change	*p*-value	*p*-value adjust	Compound	Adduct
3.62	.001403	.0114	D-Pantothenic Acid	$(M+H)^+$
3.13	.002536	.0130	Hypoxanthine	$(M+H)^+$
3.06	.000347	.0037	Citric acid	$(M-H)^-$
2.66	.000002	.0002	Citramalic acid	$(M-H)^-$
2.52	.000199	.0023	L-Phenylalanine	$(M+H)^+$
2.35	.001704	.0117	Glutamine	$(M+H)^+$
2.29	.003507	.0152	Tramadol	$(M+H-H_2O)^+$
2.28	.000026	.0006	1,4-Butynediol	$(M-H)^-$
2.22	.000022	.0006	DL-Pyroglutamic acid	$(M-H)^-$
2.10	.003257	.0144	Dehydroisoandrosterone sulfate (DHEA-S)(1)	$(M-H)^-$
2.09	.000871	.0081	5-Androsten-3-β,17-β-diol-3-sulfate	$(M-H)^-$
2.07	.000066	.0011	Glyceric acid	$(M-H)^-$
2.04	.001532	.0116	D-Ribose	$(M-H)^-$
2.04	.002385	.0130	5α-Pregnan-3α-,17-diol-20-one 3- sulfate	$(M-H)^-$
1.97	.000085	.0013	4-Hydroxyphenyllactic acid	$(M-H)^-$
1.97	.000013	.0004	L-Histidine	$(M-H)^-$
1.91	.009650	.0314	Citrulline	$(M+H)^+$
1.91	.000181	.0022	2-Hydroxy-2-methylbutyric acid	$(M-H)^-$
1.87	.000148	.0020	DL-Arginine	$(M-H)^-$
1.86	.000441	.0045	1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine	$(M-H)^-$
1.86	.005908	.0233	Androsterone sulfate	$(M-H)^-$
1.84	.001780	.0117	methyl-4-hydroxybenzoate sulfate	$(M-H)^-$
1.79	.006439	.0243	Tryptophan	$(M+H)^+$
1.77	.005997	.0233	Lauryl sulfate	$(M-H)^-$
1.76	.009494	.0314	Phe-Phe	$(M+H)^+$
1.73	.000047	.0009	Lauroyl diethanolamide	$(M+H)^+$
1.72	.000009	.0004	DL-Isocitric acid lactone	$(M-H)^-$
1.70	.003168	.0144	4-Pyridoxic acid	$(M-H)^-$
1.64	.009115	.0314	Pregnanolone sulfate	$(M-H)^-$
1.64	.009707	.0314	1,4-Diaminonaphthalene	$(M+H)^+$
1.61	.002609	.0130	Phenylacetaldehyde	$(M+H-H_2O)^+$
1.57	.001646	.0117	N,N-Dimethyldodecylamine N-oxide	$(M+H)^+$
1.56	.011574	.0347	Pipecolic acid	$(M+H)^+$
1.53	.000016	.0005	Succinic acid	$(M-H)^-$
1.52	.001329	.0113	Indolelactic acid	$(M-H)^-$
1.50	.004796	.0200	Glutamic acid	$(M-H)^-$
1.47	.007970	.0280	16-α-hydroxy DHEA 3-sulfate	$(M-H)^-$
1.47	.001836	.0117	Tegaserod	$(M+H)^+$
1.43	.010446	.0328	L-Glutamine	$(M-H)^-$
1.43	.010444	.0328	Methionine	$(M+H)^+$
1.42	.002501	.0130	L-Arginine	$(M+H)^+$
1.41	.004612	.0196	2-Indolinone	$(M+H)^+$
1.40	.009670	.0314	Nε,Nε,Nε-Trimethyl-L-lysine	$(M+H)^+$
1.33	.014215	.0408	Tyrosine	$(M+H)^+$
1.33	.002030	.0120	Methylacetate	$(M-H)^-$
1.31	.011556	.0347	Galactose	$(M-H)^-$
1.30	.006063	.0233	(E)-Chalcone	$(M+H)^+$
1.28	.002679	.0130	O-Benzyl-L-serine	$(M-H)^-$
1.28	.001982	.0120	5-Aminopentanoic acid	$(M+H)^+$
0.85	.013195	.0390	LysoPE(18:0)	$(M-H)^-$
0.83	.003137	.0144	5-Oxo-L-Proline	$(M+H)^+$
0.82	.013753	.0401	C16:0, OH FA (1) (Hydroxyhexadecanoic acid)	$(M-H)^-$
0.81	.017729	.0489	Bis(2-Ethylhexyl)Phthalate	$(M+H)^+$
0.80	.001099	.0098	Hexadecanedioic acid	$(M-H)^-$
0.78	.007669	.0274	Adipic acid	$(M-H)^-$
0.77	.007307	.0266	Phytosphingosine 1-phosphate	$(M-H_2O-H)^-$
0.77	.002823	.0134	N-Methylalanine	$(M+H)^+$
0.76	.001717	.0117	Butyrylglycine	$(M-H)^-$
0.71	.005643	.0230	D-erythro-N-stearoylsphingosine	$(M+H-H_2O)^+$
0.70	.000035	.0007	ε-Caprolactam	$(M+H)^+$
0.69	.000156	.0020	4-Nitrophenol	$(M-H)^-$
0.69	.016702	.0467	Isopropyl 4-hydroxybenzoate	$(M+H)^+$
0.69	.006539	.0243	Palmitoyl sphingomyelin	$(M+H)^+$
0.65	.000000	.0000	3,4-Dimethylbenzaldehyde	$(M+H)^+$
0.63	.002549	.0130	Indoline	$(M+H)^+$
0.63	.000100	.0015	2-Linoleoyl-1-palmitoyl-sn-glycero-3-phosphoethanolamine	$(M+H)^+$
0.61	.002060	.0120	Oleoyl-L-Carnitine	$(M+H)^+$
0.60	.016599	.0467	LysoPC(18:0)	$(M+H)^+$
0.60	.000005	.0002	DL-β-Hydroxypalmitic acid	$(M-H)^-$
0.59	.001529	.0116	5-Hydroxymethyl-2-furancarboxylic acid	$(M+CH_3CN+H)^+$
0.45	.000003	.0002	β-Hydroxymyristic acid	$(M-H)^-$
0.43	.011551	.0347	Histamine-trifluoromethyl-toluidide	$(M+H)^+$
−3.06	.000481	.0047	DL-Lactic acid	$(M-H)^-$
−32.67	.002224	.0126	3-Methyl-2-oxopentanoic acid	$(M-H)^-$

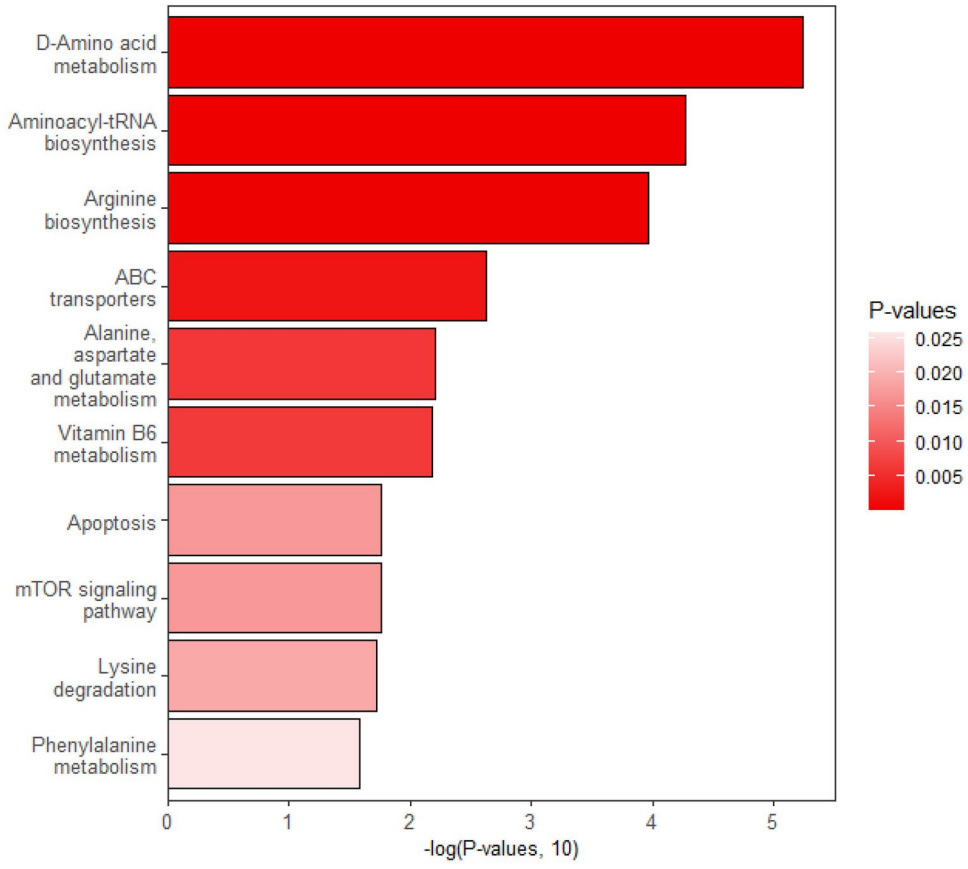

Figure 2. Pathway analysis results are presented, with the statistical significance (−log(*p*-values, 10)).

individuals, demonstrating that these changes could also be used to distinguish between skinny-fat individuals. The increasing consumption of fast food and processed foods worldwide, which contain high levels of palmitate metabolite, is believed to be contributing to the rising prevalence of NAFLD [20–22].

Citramalic acid is a metabolite found in fruits [23]. It is known that excessive fruit consumption can lead to NAFLD as it contains excessive fructose [24]. In our study, the citramalic acid metabolite was found to be high in the patient group. It is thought that this result is related to the individuals' diet.

Toledo-Ibelles et al. aimed to investigate the in-vivo relationship between NAFLD and hyperuricemia in rabbits fed a high-fat diet. They detected impaired purine metabolism due to increased hypoxanthine metabolite levels. Moreover, they demonstrated that the hypoxanthine increase was associated with increased oxidative stress in hepatocytes [25]. In addition, Ge et al. reported a decrease in fecal xanthine levels in individuals with NAFLD. In the present study, an increase in hypoxanthine metabolite was also detected. The increase in hypoxanthine metabolite in non-obese NAFLD patients suggests that this metabolite increases independent of diet.

Overall, the findings of this study provide a basis for further research on non-obese/non-overweight NAFLD patients, which is crucial for developing effective treatment options for this disease. The identification of non-invasive diagnostic biomarkers for NAFLD is urgently needed, and the insights gained from this study could contribute to the development of such biomarkers. Further studies are needed to better understand the metabolic changes associated with NAFLD and to develop targeted treatments that can improve outcomes for patients.

The primary limitation of this study was the small sample size of NAFLD in non-obese individuals, which restricted the statistical power and generalizability of the findings. To improve the reliability and robustness of the results, it is advisable to replicate the study with a larger and more representative cohort, ideally spanning diverse ethnic, demographic, and clinical subgroups. In addition, further validation of the affected pathways and mechanisms through targeted molecular investigations, such as gene expression profiling, protein quantification, or NMR-MS-based metabolomics analysis, may provide deeper insights into the underlying biology and potential therapeutic targets.

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