

Association of Nonalcoholic Fatty Liver Disease with Arterial Stiffness and its Metabolomic Profiling in Japanese Community-Dwellers

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Aims: Nonalcoholic fatty liver disease (NAFLD) is known to be associated with atherosclerosis. This study focused on upstream changes in the process by which NAFLD leads to atherosclerosis. The study aimed to confirm the association between NAFLD and the cardio-ankle vascular index (CAVI), an indicator of subclinical atherosclerosis, and explore metabolites involved in both by assessing 94 plasma polar metabolites.

Methods: A total of 928 Japanese community-dwellers (306 men and 622 women) were included in this study. The association between NAFLD and CAVI was examined using a multivariable regression model adjusted for confounders. Metabolites commonly associated with NAFLD and CAVI were investigated using linear mixed-effects models in which batch numbers of metabolite measurements were used as a random-effects variable, and false discovery rate-adjusted *p*-values were calculated. To determine the extent to which these metabolites mediated the association between NAFLD and CAVI, mediation analysis was conducted.

Results: NAFLD was positively associated with CAVI (coefficients [95% Confidence intervals (CI)]=0.23 [0.09–0.37]; *p*=0.001). A total of 10 metabolites were involved in NAFLD and CAVI, namely, branched-chain amino acids (BCAAs; valine, leucine, and isoleucine), aromatic amino acids (AAAs; tyrosine and tryptophan), alanine, proline, glutamic acid, glycerophosphorylcholine, and 4-methyl-2-oxopentanoate. Mediation analysis showed that BCAAs mediated more than 20% of the total effect in the association between NAFLD and CAVI.

Conclusions: NAFLD was associated with a marker of atherosclerosis, and several metabolites related to insulin resistance, including BCAAs and AAAs, could be involved in the process by which NAFLD leads to atherosclerosis.

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Key words: Nonalcoholic fatty liver disease, Cardio-ankle vascular index, Atherosclerosis, Metabolites, Branched-chain amino acids, Insulin resistance

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common type of chronic liver disease worldwide,

with an estimated prevalence of 20%–30% in the general population¹⁾. Fatty liver is caused by accumulation of triglycerides that subsequently form lipid droplets in the liver. Fat accumulation has been

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associated with enhanced free fatty acid flux to the liver, increased hepatic *de novo* lipogenesis, impaired mitochondrial β -oxidation, and decreased hepatic lipid secretion via very-low-density lipoprotein^{2, 3}). Increased accumulation of these lipids in the liver contributes to lipotoxicity, hepatic insulin resistance (IR), increased inflammatory response, and oxidative stress^{2, 3}).

NAFLD has been closely associated with the development of cardiometabolic risk factors, such as diabetes and hypertension^{4, 5}). Furthermore, previous studies have reported that NAFLD was associated with subclinical atherosclerosis, such as carotid artery atherosclerosis and arterial stiffness⁶). Although several possible mechanisms are caused by fat accumulation, as aforementioned, they remain poorly understood. Given that the liver plays a pivotal role in multiple metabolic pathways, it is essential to perceive changes as early as possible to predict subsequent disease for the prevention. For this reason, previous metabolomics studies have identified metabolites related to NAFLD⁷).

Few studies have explored the metabolites involved in the association between NAFLD and atherosclerosis within the context of one study while performing metabolic profiling, a comprehensive assessment of metabolites, to speculate upstream changes in the process by which NAFLD leads to atherosclerosis. This cross-sectional study was conducted in Japanese community-dwellers to confirm the association between NAFLD and cardio-ankle vascular index (CAVI), an indicator of subclinical atherosclerosis⁸), and to explore metabolites involved in both by assessing 94 plasma polar metabolites via capillary electrophoresis–mass spectrometry (CE-MS).

Methods

Study Participants

The Tsuruoka Metabolomics Cohort Study (TMCS) is a population-based observational study in Tsuruoka City, Yamagata Prefecture, Japan. The study participants were recruited from individuals involved in health check-up programs implemented by Tsuruoka City or employers. The baseline survey was conducted between fiscal years 2012 and 2015, which included 11,002 participants aged 35–74 years. Details regarding the TMCS have been described elsewhere^{9–13}). The study protocol was approved by the Medical Ethics Committee of the School of Medicine, Keio University, Tokyo, Japan (approval no. 20110264).

Health check-up programs are implemented by Tsuruoka City at several facilities in the city, and some

optional examination items in these programs are provided depending on the facility. This cross-sectional study included 1,491 participants who received the health check-up provided by Tsuruoka City in the facility where CAVI and abdominal ultrasound examination were performed at baseline. Among them, 563 were excluded due to missing data ($n=7$), history of cardiovascular disease (CVD) ($n=57$), history of cancer ($n=153$), hepatitis B or C virus ($n=30$), liver cirrhosis ($n=1$), nonfasting state on blood sampling ($n=7$), alcohol consumption beyond the NAFLD criteria (30 and 20 g/day in men and women, respectively; $n=300$)¹⁴), and ankle brachial index (ABI) <0.9 ($n=8$)¹⁵). Ultimately, 928 participants (306 men and 622 women) were included in the final analysis.

Examinations

Body mass index (BMI) was calculated by dividing weight (kg) by height (m²). Blood pressure was measured twice in the sitting position using an automated sphygmomanometer (Omron HBP-T105S-N). Blood samples were collected in a fasted state 10 h after the participants' last meal. Serum total cholesterol (TC) and triglycerides (TG) were measured enzymatically, whereas high-density lipoprotein cholesterol (HDL-C) was measured using a direct enzymatic method. Non-HDL-C was calculated by subtracting HDL-C from TC. Smoking and drinking status, medication, and disease history were evaluated using standard self-report questionnaires and validated by well-trained interviewers. The levels of fasting plasma glucose and hemoglobin A1c (HbA1c) were measured employing enzymatic methods and high-performance liquid chromatography, respectively. Furthermore, gamma-glutamyl transpeptidase (γ -GTP) was measured via colorimetry, whereas aspartate and alanine aminotransferases were measured employing ultraviolet spectrophotometry and the Japanese Society of Clinical Chemistry standardization method.

Hypertension was defined as systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg, or taking antihypertensive medications¹⁶). Diabetes was defined as HbA1c (NGSP) $\geq 6.5\%$, fasting plasma glucose ≥ 126 mg/dL, or taking medications for diabetes¹⁷). Dyslipidemia was defined as TG ≥ 150 mg/dL, non-HDL-C ≥ 170 mg/dL, HDL-C <40 mg/dL, or taking lipid-lowering medications¹⁸).

NAFLD was defined as fatty liver detected via ultrasonography (ProSound SSD-5500 or ProSound Alpha 5, ALOKA, Tokyo, Japan) in the absence of other causes of chronic liver disease (i.e., no history of

hepatitis C and B virus infection, alcohol consumption <30 g/day for men and <20 g/day for women¹⁴⁾). In ultrasonography, fatty liver was diagnosed based on the following findings: bright liver, increased liver–kidney (spleen) contrast, blurring of blood vessels, and deep attenuation of the liver¹⁹⁾. Furthermore, metabolic dysfunction-associated fatty liver disease (MAFLD) was defined as fatty liver with diabetes, fatty liver with BMI ≥ 23 kg/m², or fatty liver with BMI <23 kg/m² and the presence of at least two metabolic risk abnormalities from the following: 1) waist circumference ≥ 90 cm for men and ≥ 80 cm for women, 2) blood pressure $\geq 130/85$ mmHg or taking medication for hypertension, 3) HDL-C <40 mg/dL for men and <50 mg/dL for women or taking specific medication, 4) TG ≥ 150 mg/dL or taking specific medication, 5) prediabetes: fasting glucose 100–125 mg/dL or HbA1c 5.7% to 6.4%²⁰⁾. CAVI was measured in the supine position using a VaSera (VS-1500AN) CAVI instrument (Fukuda Denshi Co. Ltd., Tokyo, Japan), and the value was obtained by measuring blood pressure and pulse-wave velocity. Arterial stiffness was defined as CAVI ≥ 9.0 ^{21, 22)}. Simultaneously, ABI was estimated by measuring the blood pressure of the brachial and tibial arteries.

Sample Collection and Measurement of Metabolites

To prepare optimal samples for the CE-MS metabolomics platform, appropriate protocols were followed as previously reported^{9, 23)}. Briefly, blood samples were collected in the morning after a 12-h overnight fast. Plasma was stored at 4°C immediately after collection using ethylenediaminetetraacetic acid-2Na as the anticoagulant. Samples were then centrifuged (1,500 g, 4°C) for 15 min within 3 h after collection, divided into aliquots, and stored at 4°C until metabolite extraction. Metabolite extraction from plasma was completed within 6 h after collection to reduce metabolic reactions in the plasma, after which the extracts were stored at –80°C. For the sample extraction, 50 μ L of plasma was used. Details regarding the extraction method have been described elsewhere²⁴⁾.

Metabolomic profiling was performed using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS). CE-TOFMS analysis of cationic and anionic metabolites was conducted. Details regarding the profiling method have been described elsewhere^{25–28)}. For all samples, the absolute concentrations of 94 metabolites (54 cations and 40 anions) expected to be detected in at least 20% of the plasma samples were measured. These data consist of 105 and 99 running batches of cations and anions,

respectively. Each batch included an average of 80.1 samples (maximum 164) for cations and 83.2 samples (maximum 168) for anions, respectively. To monitor the stability of the metabolomics analysis, quality control (QC) samples were measured every 10 samples and evaluated at the beginning of the analysis and at intervals throughout the analysis. QC samples were obtained from 150 mL of serum collected in advance from 20 individuals among the same population, extracted for metabolomic analysis immediately after collection, divided into 50- μ L aliquots, and stored at –80°C. QC aliquots stored at –80°C were thawed and used for daily monitoring throughout the study period. The mean concentration of each metabolite in the QC samples previously analyzed in 70 sequences was calculated. If the concentration of each metabolite in a QC sample exceeded the mean concentration ± 2 standard deviations (SDs) for more than half of the metabolites consecutively (more than twofold), the samples in subsequent sequences were reanalyzed. The reliability of these metabolite measurements has been validated²⁵⁾.

Statistical Analysis

The study participants were divided into two groups according to the presence or absence of NAFLD. Continuous variables with a normal distribution were expressed as mean values and SDs, whereas those with a skewed distribution were presented as medians and interquartile ranges. Categorical variables were expressed as numbers and proportions according to groups. In addition, these were presented similarly according to the participants who did or did not undergo CAVI measurement and ultrasound among the overall TMCS population after excluding those with missing data. Continuous variables were compared in the two populations using the unpaired *t*-test or the Mann–Whitney *U* test. On the other hand, categorical variables were compared using the χ^2 test.

Multivariable regression analysis was conducted to determine the association between NAFLD and increased CAVI values. In addition, multivariable logistic analysis was conducted to estimate the odds ratio of NAFLD for arterial stiffness (CAVI ≥ 9.0). In these analyses, Model 1 was adjusted for sex and age; Model 2, for the variables in Model 1 plus BMI, smoking status (never, past, and current smokers), and alcohol-drinking status (never, past, and current drinkers); and Model 3, for the variables in Model 2 plus diabetes, hypertension, and dyslipidemia.

To identify metabolites commonly related to NAFLD and CAVI, analysis was conducted as follows, with all metabolites being log-transformed. First, the

Table 1. Characteristics of the study participants

	Non-NAFLD	NAFLD	Total
No. of participants	671	257	928
Men, <i>n</i> (%)	192 (28.6%)	114 (44.3%)	306 (32.9%)
Age, years	65.1 ± 6.2	64.3 ± 7.1	64.9 ± 6.4
Body mass index, kg/m ²	22.5 ± 2.9	26.0 ± 3.3	23.5 ± 3.4
AST, U/L	21 [18–24]	23 [19–29]	21 [18–25]
ALT, U/L	16 [13–20]	24 [18–35]	18 [14–23]
γ-GTP, U/L	19 [14–27]	29 [19–44]	21 [15–33]
Hypertension, <i>n</i> (%)	278 (41.4%)	137 (53.3%)	415 (44.7%)
Diabetes, <i>n</i> (%)	45 (6.7%)	52 (20.2%)	97 (10.4%)
Dyslipidemia, <i>n</i> (%)	257 (38.3%)	151 (58.7%)	408 (43.9%)
Smoking status, <i>n</i> (%)			
Never smoker	496 (73.9%)	159 (61.9%)	655 (70.6%)
Ex-smoker	136 (20.3%)	80 (31.1%)	216 (23.3%)
Current smoker	39 (5.8%)	18 (7.0%)	57 (6.1%)
Alcohol-drinking status, <i>n</i> (%)			
Never drinker	413 (61.6%)	151 (58.8%)	564 (60.8%)
Ex-drinker	52 (7.7%)	19 (7.3%)	71 (7.7%)
Current drinker	206 (30.7%)	87 (33.9%)	293 (31.5%)
MAFLD, <i>n</i> (%)	-	242 (94.1%)	242 (26.0%)

Data are presented as mean ± standard deviation for continuous variables or median [interquartile range].

NAFLD, nonalcoholic fatty liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyltransferase; MAFLD, metabolic dysfunction-associated fatty liver disease

coefficients of metabolites from non-NAFLD to NAFLD calculated using a linear mixed-effects model with log-transformed metabolites as the dependent variable and batch numbers of metabolite measurements as a random-effects variable were back transformed to estimate the fold changes in metabolites from non-NAFLD to NAFLD. False discovery rate (FDR)-adjusted *p*-values were calculated to control for the proportion of false-positive results. The fold change represents the ratio of metabolite concentrations from non-NAFLD to NAFLD^{9–11, 29, 30}. Thereafter, for metabolites with FDR-adjusted *p*-values < 0.05 in the previous analysis, coefficients per 1 SD of log-transformed metabolites for increased CAVI values were estimated using linear mixed-effects models with batch numbers of metabolite measurements as a random-effects variable. In these analyses, sex, age, BMI, smoking status (never, past, and current smokers), and alcohol-drinking status (never, past, and current drinkers) were included as adjustment variables, and the FDR-adjusted *p*-values were calculated. Further analyses were conducted, adding diabetes, hypertension, and dyslipidemia as adjustment variables.

To determine the extent to which metabolites observed both in NAFLD and increased CAVI values mediated the association between NAFLD and

increased CAVI values, mediation analysis based on a multivariable regression model adjusting for sex, age, BMI, smoking status (never, past, and current smokers), and alcohol-drinking status (never, past, and current drinkers) was conducted using the Stata procedures proposed by R. Hicks and D. Tingley³¹. In the analysis, coefficients for indirect and total effects and the percentage of the total effect mediated by metabolites in the association between NAFLD and CAVI values were estimated.

Confidence intervals (CIs) were estimated at the 95% level. Statistical analyses were conducted using STATA/SE 17 data analysis and statistical software (Stata Corp LP, College Station, TX, USA).

Results

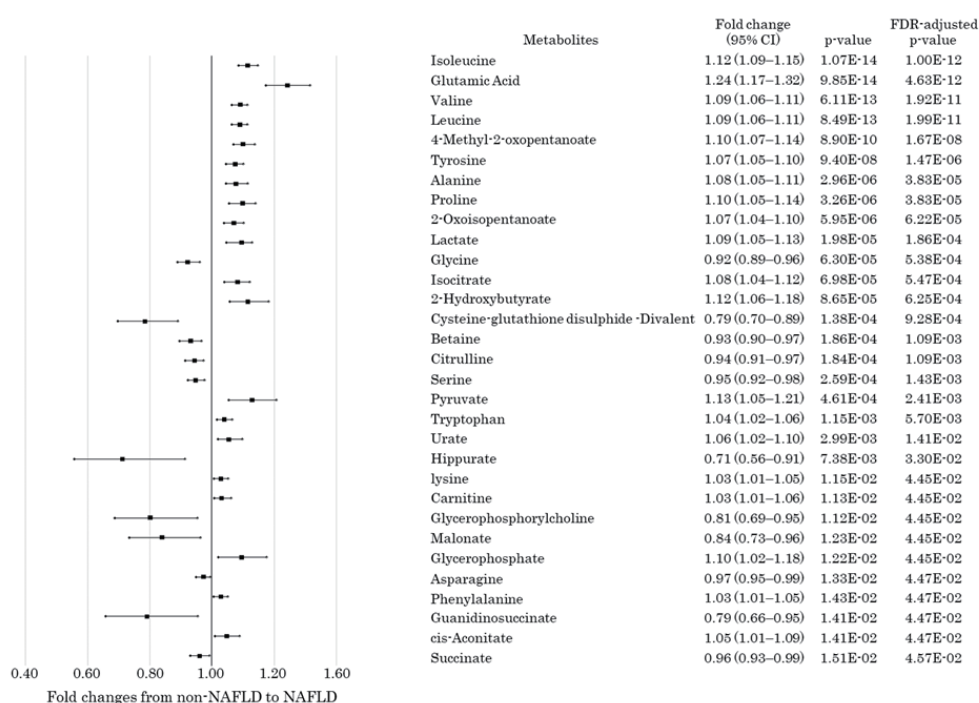
The characteristics of the study participants with and without NAFLD are presented in **Table 1**. BMI, liver enzyme levels, and prevalence of cardiometabolic risk factors, such as diabetes, hypertension, and dyslipidemia, were higher in the NAFLD group than in the non-NAFLD group. The proportion of current smokers and drinkers seemed to be slightly higher in the NAFLD group than in the non-NAFLD group. The characteristics of participants who did or did not undergo CAVI measurement and abdominal

Table 2. Association between NAFLD and CAVI values and arterial stiffness

	CAVI values		Arterial stiffness	
	Coefficient (95% CI)	<i>p</i> value	Odds ratio (95% CI)	<i>p</i> value
Model 1	0.08 (−0.04–0.20)	0.21	1.71 (1.17–2.50)	0.005
Model 2	0.23 (0.09–0.37)	0.001	2.30 (1.49–3.55)	<0.001
Model 3	0.19 (0.05–0.33)	0.007	2.07 (1.31–3.27)	0.002

NAFLD, nonalcoholic fatty liver disease; CAVI, Cardiac Ankle Vascular Index; 95% CI, 95% confidence interval

Coefficients were calculated using multivariable regression models with NAFLD as an explanatory variable and CAVI as an objective variable. Odds ratios were calculated using multivariable logistic models with NAFLD as an explanatory variable and arterial stiffness as an objective variable. Arterial stiffness was defined as CAVI ≥ 9.0 . Model 1 was adjusted for sex and age. Model 2 was adjusted for sex, age, body mass index, smoking status, and alcohol-drinking status. Model 3 was adjusted for sex, age, body mass index, smoking status, alcohol-drinking status, diabetes, hypertension, and dyslipidemia.

**Fig. 1.** Association between NAFLD and metabolites

FDR, false discovery rate; NAFLD, nonalcoholic fatty liver disease

All metabolites were log-transformed. Fold changes in metabolites from non-NAFLD to NAFLD were estimated using linear mixed-effects model with batch numbers of metabolite measurements as a random-effects variable. The model was adjusted for sex, age, body mass index, smoking status (never, past, and current smokers), and alcohol-drinking status (never, past, and current drinkers). Metabolites with FDR-adjusted *p*-values <0.05 are shown.

ultrasound examination among the overall TMCS population are presented in [Supplemental Table 1](#). Participants who underwent these examinations were older and had a higher prevalence of hypertension and diabetes than those who did not.

The coefficients and odds ratios for the association between NAFLD and CAVI values and arterial stiffness (CAVI ≥ 9.0), respectively, are presented in [Table 2](#). After adjusting for confounders, including BMI, NAFLD was positively associated

with CAVI values. The odds ratios for arterial stiffness were higher in NAFLD. These results were observed even after adjusting for cardiometabolic risk factors.

The fold changes in metabolites from non-NAFLD to NAFLD are detailed in [Fig. 1](#) (the results for all metabolites are presented in [Supplemental Table 2](#)). Among the 94 metabolites analyzed, 31 exhibited an FDR-adjusted *p*-value <0.05. The metabolites associated with NAFLD were branched-chain amino acids (BCAAs; valine, leucine, and

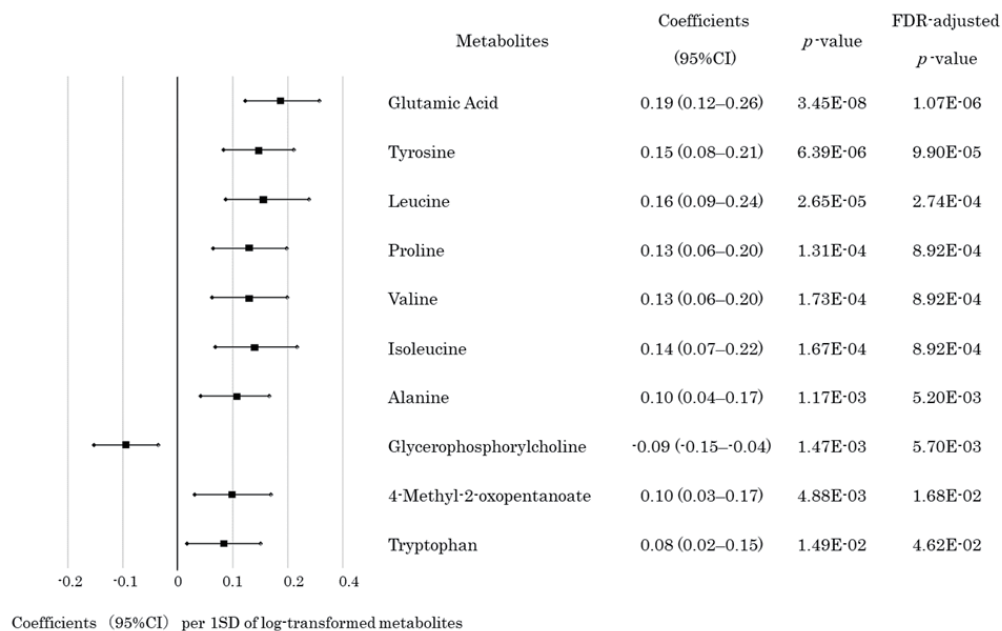


Fig. 2. Association between metabolites and the cardio-ankle vascular index

FDR, false discovery rate

For 31 metabolites with FDR-adjusted p -values <0.05 in the previous analysis, coefficients per 1 SD of log-transformed metabolites for increased CAVI values were estimated using linear mixed-effects model with batch numbers of metabolite measurements as a random-effects variable. The model was adjusted for sex, age, body mass index, smoking status (never, past, and current smokers), and alcohol-drinking status (never, past, and current drinkers). Metabolites with FDR-adjusted p -values <0.05 are shown.

isoleucine), aromatic amino acids (AAAs; phenylalanine, tryptophan, and tyrosine), and substances related to the metabolism. Metabolites related to glutathione metabolism, the glycolytic pathway, and the tricarboxylic acid cycle were also detected.

Among the metabolites detected in NAFLD, those associated with CAVI values are presented in **Fig. 2** (the results for the 31 metabolites detected in the previous analysis are presented in **Supplemental Table 3**). Coefficients per 1 SD of log-transformed metabolites were presented. Overall, 10 metabolites exhibited an FDR-adjusted p -value <0.05 . The observed metabolites included BCAAs (valine, leucine, and isoleucine), AAAs (tyrosine and tryptophane), alanine, proline, glutamic acid, glycerophosphorylcholine, and 4-methyl-2-oxopentanoate

Supplemental Tables 4 and 5 present the fold changes in metabolites from non-NAFLD to NAFLD and coefficients of metabolites associated with CAVI, respectively, after adding diabetes, hypertension, and dyslipidemia to the adjustment variables. Among the 94 metabolites analyzed, 20 exhibited an FDR-adjusted p -value <0.05 , and of these 20 metabolites, 8, including BCAA and AAA, were associated with CAVI values.

The coefficients for indirect and total effects and

the percentage of total effect mediated by metabolites in the association between NAFLD and CAVI during the mediation analysis are presented in **Table 3**. BCAAs were observed to mediate over 20% of the total effect in the association between NAFLD and CAVI. Glutamic acid had the highest mediated effect (37.0%).

Discussion

The present study demonstrated that NAFLD was associated with increased CAVI values, which serves as an indicator of arterial stiffness. This result is consistent with the findings of previous studies⁶. In addition, detailed metabolomic profiling revealed that NAFLD was associated with 31 metabolites. Among them, elevated concentrations of BCAAs (valine, leucine, and isoleucine); AAAs (tyrosine and tryptophan); proline; glutamic acid, which is produced as the primary decomposition reaction of BCAA; and 4-methyl-2-oxopentanoate, which is synthesized from leucine in the reaction, were also found to be involved in CAVI values. Mediation analysis revealed that these BCAAs and AAAs, as well as the related metabolites, may mediate the association between NAFLD and atherosclerosis.

Previous studies have also demonstrated that

Table 3. Mediation analysis of the association between non-alcoholic fatty liver disease and the Cardiac Ankle Vascular Index

Mediator	Mediated effect Coefficient (95%CI)	Direct effect coefficient (95%CI)	Proportion of total effect mediated % (95%CI)
4-Methyl-2-oxopentanoate	0.02 (0.01–0.05)	0.20 (0.06–0.34)	12.5 (7.7–29.3)
Alanine	0.02 (0.01–0.05)	0.20 (0.06–0.34)	12.0 (7.4–28.0)
Glutamic acid	0.08 (0.04–0.12)	0.14 (0.01–0.28)	37.0 (22.3–85.8)
Glycerophosphorylcholine	0.01 (–0.001–0.03)	0.21 (0.08–0.35)	5.4 (3.4–12.6)
Isoleucine	0.05 (0.01–0.09)	0.17 (0.03–0.32)	23.2 (14.0–54.8)
Leucine	0.05 (0.02–0.09)	0.17 (0.03–0.31)	25.0 (15.2–59.6)
Proline	0.03 (0.01–0.06)	0.19 (0.06–0.33)	15.3 (9.4–35.7)
Tryptophan	0.01 (0.001–0.03)	0.21 (0.08–0.35)	6.8 (4.2–16.1)
Tyrosine	0.04 (0.02–0.08)	0.18 (0.04–0.32)	21.3 (13.0–50.1)
Valine	0.04 (0.01–0.08)	0.18 (0.04–0.32)	21.4 (13.0–51.0)

Mediation analysis based on a multivariable regression model was performed adjusting for sex, age, body mass index, smoking status (never, past, and current smokers), and alcohol-drinking status (never, past, and current drinkers). Coefficient for indirect and total effects and the percentage of the total effect mediated by each metabolite in the association between NAFLD and CAVI values were estimated.

BCAAs and AAAs were elevated in NAFLD^{7, 32, 33)} and that these substances were associated with IR. Notably, previous interventional studies have reported that BCAA intake from diet and supplements often improves IR, diabetes risk, and liver disease risk³⁴⁾. Meanwhile, observational studies have demonstrated that elevated levels of BCAAs increase the risk of developing IR and diabetes³⁵⁾. The multiethnic Insulin Resistance Atherosclerosis Study, for instance, suggests that elevated plasma BCAAs were inversely associated with insulin sensitivity and metabolic clearance of insulin and positively associated with incident diabetes. Similarly, the population-based Cardiovascular Risk in Young Finns Study reported that BCAAs and AAAs predict 6-year IR in young adults³⁶⁾. Unfortunately, the underlying molecular mechanism for the causal association of BCAAs and AAAs with IR remains unclear. Nonetheless, two possible mechanisms have been proposed. One is that increases in BCAAs due to impaired catabolism activate the mammalian target of the rapamycin complex 1 (mTORC1) signaling pathway, and persistent signaling might cause IR through serine phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2³⁷⁾. The other proposed mechanism is that BCAAs do not directly cause IR but that metabolic intermediates produced by elevated BCAAs induce mitochondrial dysfunction and mitochondrial oxidative stress, leading to IR^{37, 38)}. These mechanisms might explain the findings obtained in the present study.

The present study also demonstrated that BCAAs were associated with increased CAVI values. The association between BCAAs and subclinical atherosclerosis and CVD could be determined based

on glycemic status and the presence of diabetes. A previous study reported that plasma BCAAs were positively associated with carotid intima-media thickness among individuals with HbA1c $\geq 5.6\%$ but not among those with HbA1c $< 5.6\%$ ³⁹⁾. Furthermore, the Women's Health Study found that BCAAs were positively associated with CVD during a mean follow-up period of 18.6 years (hazard ratio per 1 SD: 1.13 [95% CI 1.08–1.18]) and that an interaction existed between BCAAs and the presence of diabetes for incident CVD (p for interaction = 0.036; women with diabetes: HR per 1 SD, 1.20 [95% CI 1.08–1.32]; women without diabetes: HR per 1 SD, 1.08 [95% CI 1.03–1.14])⁴⁰⁾. Furthermore, a Mendelian randomization study reported that elevated levels of BCAAs caused IR and diabetes⁴¹⁾. As for the mechanism, elevated BCAA levels are known to generate inflammation and oxidative stress in endothelial cells, promoting inflammatory cell adhesion and endothelial dysfunction^{42, 43)}. Given the findings of these previous observational studies, BCAAs and AAAs in NAFLD may be considered upstream markers of atherosclerosis prior to IR or diabetes. In particular, the fact that 94% of patients with NAFLD in the present study met the MAFLD criteria suggests that some metabolic abnormalities related to IR might have already occurred among most of those with NAFLD. Accordingly, the present study found that these preceding changes may be present in the process by which NAFLD leads to atherosclerosis.

The present study demonstrated that individuals with NAFLD had alterations in several amino acids, such as BCAAs, AAAs, glutamic acid, proline, alanine, and glycine. The level of cysteine-glutathione disulfide, a product of glutathione and cysteine

conjugate, was found to decrease in the NAFLD group. The levels of glycine and serine, which are used in the synthesis of glutathione, also decreased in the same group. These findings are consistent with those of previous studies^{7, 44}). As aforementioned, BCAAs and AAAs are strongly associated with IR³⁵). Meanwhile, glutathione is the major antioxidant in the liver that is biosynthesized in response to oxidative stress. In NAFLD, fat accumulation in the liver causes oxidative stress, leading to the generation of reactive oxygen species that could potentially exhaust the supply of antioxidants, such as glutathione. This could result in a decrease in the concentration of cysteine-glutathione disulfide, an oxidation product of glutathione^{44, 45}). Such dysregulated glutathione homeostasis in NAFLD could also be associated with the regulation of signaling pathways involved in inflammation⁴⁶). Thus, the decrease in cysteine-glutathione disulfide levels in NAFLD may contribute to the development and progression of liver inflammation and damage. The present study found changes in metabolites associated with these NAFLD-specific biological responses

This study has some limitations. First, given the cross-sectional design of this study, causal relationships could not be definitively confirmed. However, previous studies have also suggested a causal relationship between fatty liver and atherosclerosis, which could support our findings on causality. Second, some factors can affect the measurement variability of metabolomic analysis, leading to the misclassification of metabolomics data. To minimize these variations, the fasting conditions for participants and the QC procedures for metabolomics analysis were standardized. Finally, generalizability was limited as the study population consisted of residents from only one region in Japan. In addition, the prevalence of atherosclerosis due to aging and diabetes may have been higher in this study population than in the overall TMCS population. For this reason, the association between fatty liver and atherosclerosis in the study population may have been more apparent than the association in the overall TMCS population.

In conclusion, the present study found an association between NAFLD and subclinical atherosclerosis in Japanese community-dwellers. Furthermore, we found that metabolites associated with IR might be involved in the relationship. This finding suggests that alterations in these metabolites may indicate upstream changes in the association between NAFLD and atherosclerosis, indicating that early management of IR in NAFLD could prevent the development of atherosclerosis.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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Supplemental Table 1. Characteristics of participants with and without receiving CAVI and abdominal ultrasound examination among the overall TMCS population

	Participants with receiving CAVI and abdominal ultrasound (<i>n</i> = 1,489)	Participants without receiving CAVI and abdominal ultrasound (<i>n</i> = 9,436)	Total (<i>n</i> = 10,925)	<i>p</i> -value
Men, <i>n</i> (%)	717 (48.1%)	4,376 (46.3%)	5,093 (46.6%)	0.20
Age, years	64.9 ± 6.6	58.7 ± 10.2	59.6 ± 10.1	< 0.001
Body mass index, kg/m ²	23.4 ± 3.3	23.2 ± 3.3	23.2 ± 3.3	0.005
AST, U/L	22 [19-26]	22 [18-26]	22 [18-26]	0.02
ALT, U/L	19 [14-25]	18 [14-25]	18 [14-25]	0.12
γ-GTP, U/L	24 [16-42]	24 [16-42]	24 [16-42]	0.77
Hypertension, <i>n</i> (%)	753 (50.5%)	4,046 (42.8%)	4,799 (43.9%)	< 0.001
Diabetes, <i>n</i> (%)	196 (13.1%)	961 (10.1%)	1,157 (10.5%)	0.001
Dyslipidemia, <i>n</i> (%)	645 (43.3%)	3,978 (42.1%)	4,623 (42.3%)	0.40
Smoking status, <i>n</i> (%)				
Never-smoker	844 (56.7%)	5,275 (55.9%)	6,119 (56.0%)	< 0.001
Ex-smoker	504 (33.8%)	2,653 (28.1%)	3,157 (28.9%)	
Current-smoker	141 (9.5%)	1,508 (16.0%)	1,649 (15.1%)	
Alcohol-drinking status, <i>n</i> (%)				
Never-drinker	675 (45.3%)	4,142 (43.9%)	4,817 (44.1%)	0.20
Ex-drinker	92 (6.2%)	512 (5.4%)	604 (5.5%)	
Current-drinker	722 (48.5%)	4,782 (50.7%)	5,504 (50.4%)	

Individuals who had missing data of the variables presented in this table were excluded.

Continuous variables were compared in the population that received CAVI and abdominal ultrasound versus the population that did not using the unpaired *t*-test or the Mann-Whitney *U* test. Categorical variables were compared using the χ^2 test.

Supplemental Table 2. Association between NAFLD and metabolites (results for all metabolites)

Metabolites	Fold change	95%confidence interval		<i>p</i> -value	FDR-adjusted <i>p</i> -value
		lower	upper		
Isoleucine	1.12	1.09	1.15	1.07E-14*	1.00E-12*
Glutamic Acid	1.24	1.17	1.32	9.85E-14*	4.63E-12*
Valine	1.09	1.06	1.11	6.11E-13*	1.92E-11*
Leucine	1.09	1.06	1.11	8.49E-13*	1.99E-11*
4-Methyl-2-oxopentanoate	1.10	1.07	1.14	8.90E-10*	1.67E-08*
Tyrosine	1.07	1.05	1.10	9.40E-08*	1.47E-06*
Alanine	1.08	1.05	1.11	2.96E-06*	3.83E-05*
Proline	1.10	1.05	1.14	3.26E-06*	3.83E-05*
2-Oxoisopentanoate	1.07	1.04	1.10	5.95E-06*	6.22E-05*
Lactate	1.09	1.05	1.13	1.98E-05*	1.86E-04*
Glycine	0.92	0.89	0.96	6.30E-05*	5.38E-04*
Isocitrate	1.08	1.04	1.12	6.98E-05*	5.47E-04*
2-Hydroxybutyrate	1.12	1.06	1.18	8.65E-05*	6.25E-04*
Cysteine-glutathione disulphide -Divalent	0.79	0.70	0.89	1.38E-04*	9.28E-04*
Betaine	0.93	0.90	0.97	1.86E-04*	1.09E-03*
Citrulline	0.94	0.91	0.97	1.84E-04*	1.09E-03*
Serine	0.95	0.92	0.98	2.59E-04*	1.43E-03*
Pyruvate	1.13	1.05	1.21	4.61E-04*	2.41E-03*
Tryptophan	1.04	1.02	1.06	1.15E-03*	5.70E-03*
Urate	1.06	1.02	1.10	2.99E-03*	1.41E-02*
Hippurate	0.71	0.56	0.91	7.38E-03*	3.30E-02*
Lysine	1.03	1.01	1.05	1.15E-02*	4.45E-02*
Carnitine	1.03	1.01	1.06	1.13E-02*	4.45E-02*
Glycerophosphorylcholine	0.81	0.69	0.95	1.12E-02*	4.45E-02*
Malonate	0.84	0.73	0.96	1.23E-02*	4.45E-02*
Glycerophosphate	1.10	1.02	1.18	1.22E-02*	4.45E-02*
Asparagine	0.97	0.95	0.99	1.33E-02*	4.47E-02*
Phenylalanine	1.03	1.01	1.05	1.43E-02*	4.47E-02*
Guanidinosuccinate	0.79	0.66	0.95	1.41E-02*	4.47E-02*
cis-Aconitate	1.05	1.01	1.09	1.41E-02*	4.47E-02*
Succinate	0.96	0.93	0.99	1.51E-02*	4.57E-02*
Mucate	0.96	0.92	1.00	4.05E-02*	1.19E-01
gamma-Butyrobetaine	0.97	0.94	1.00	4.30E-02*	1.22E-01
Choline	0.97	0.94	1.00	4.44E-02*	1.23E-01
Histidine	1.02	1.00	1.04	5.33E-02	1.43E-01
Creatine	1.06	0.99	1.13	8.79E-02	2.18E-01
trans-Aconitate	0.89	0.78	1.02	8.80E-02	2.18E-01
Glucuronate	1.07	0.99	1.16	8.37E-02	2.18E-01
Threonine	0.98	0.95	1.00	1.01E-01	2.44E-01
N-Acetylaspartate	0.97	0.92	1.01	1.14E-01	2.61E-01
Citrate	0.97	0.94	1.01	1.12E-01	2.61E-01
Trimethylamine N-oxide	0.90	0.78	1.04	1.44E-01	2.88E-01
Sarcosine	0.90	0.78	1.04	1.44E-01	2.88E-01
Trigonelline	0.81	0.61	1.08	1.47E-01	2.88E-01
proline betaine	0.88	0.74	1.04	1.42E-01	2.88E-01
Methionine	1.02	0.99	1.06	1.42E-01	2.88E-01
2-Oxobutyrate	0.88	0.73	1.04	1.38E-01	2.88E-01
threonate	0.97	0.94	1.01	1.45E-01	2.88E-01
Quinate	0.85	0.67	1.07	1.62E-01	3.11E-01

(Cont. Supplemental Table 2)

Metabolites	Fold change	95%confidence interval		<i>p</i> -value	FDR-adjusted <i>p</i> -value
		lower	upper		
3-Indoxyl sulfate	1.09	0.96	1.22	1.85E-01	3.47E-01
o-Acetylcarnitine	1.07	0.96	1.19	2.00E-01	3.69E-01
alpha-Aminoadipate	1.09	0.95	1.26	2.23E-01	4.00E-01
4-Acetylbutyrate	1.09	0.95	1.26	2.26E-01	4.00E-01
Guanidinoacetate	0.98	0.94	1.02	2.49E-01	4.12E-01
Aspartic acid	1.06	0.96	1.16	2.55E-01	4.12E-01
Glutamine	1.01	0.99	1.03	2.49E-01	4.12E-01
Cystine	1.01	0.99	1.04	2.45E-01	4.12E-01
Isethionate	0.97	0.91	1.02	2.52E-01	4.12E-01
3-Methylhistidine	1.05	0.96	1.15	3.06E-01	4.87E-01
3-Hydroxybutyrate	0.95	0.85	1.05	3.17E-01	4.88E-01
Fumarate	1.04	0.96	1.12	3.15E-01	4.88E-01
Pipecolate	0.97	0.90	1.04	3.47E-01	5.27E-01
Arginine	0.99	0.96	1.02	4.01E-01	5.98E-01
Citraconate	0.93	0.76	1.14	4.70E-01	6.90E-01
ADMA	0.95	0.83	1.09	5.01E-01	7.20E-01
Uridine	0.99	0.97	1.01	5.05E-01	7.20E-01
Creatinine	0.99	0.97	1.02	5.13E-01	7.20E-01
Pelargonate	1.01	0.97	1.06	5.23E-01	7.23E-01
Ornithine	1.01	0.98	1.05	5.34E-01	7.28E-01
Glutarate	1.02	0.95	1.09	5.63E-01	7.56E-01
5-Oxoproline	0.99	0.97	1.02	5.94E-01	7.87E-01
Heptanoate	0.97	0.86	1.09	6.11E-01	7.98E-01
SDMA	0.98	0.86	1.11	7.10E-01	9.02E-01
Cysteine S-sulfate	0.98	0.90	1.07	7.06E-01	9.02E-01
beta-Ala	1.01	0.94	1.09	7.50E-01	9.41E-01
3-Aminoisobutyrate	0.99	0.83	1.17	8.72E-01	9.50E-01
2AB	1.00	0.95	1.04	8.53E-01	9.50E-01
N,N-Dimethylglycine	1.00	0.95	1.06	8.77E-01	9.50E-01
Hydroxyproline	1.01	0.96	1.06	7.94E-01	9.50E-01
Triethanolamine	0.97	0.77	1.21	7.78E-01	9.50E-01
Indole-3-acetate	0.98	0.82	1.17	8.44E-01	9.50E-01
Kynurenine	1.01	0.92	1.11	8.40E-01	9.50E-01
Malate	1.00	0.96	1.04	8.89E-01	9.50E-01
Octanoate	0.99	0.88	1.12	8.79E-01	9.50E-01
2-Oxoglutarate	0.99	0.93	1.05	8.08E-01	9.50E-01
Terephthalate	1.00	0.96	1.05	8.87E-01	9.50E-01
Decanoate	1.01	0.94	1.08	8.22E-01	9.50E-01
Azelate	0.99	0.94	1.05	7.77E-01	9.50E-01
Taurine	1.00	0.97	1.03	9.12E-01	9.53E-01
1-Methylnicotinamide	0.99	0.83	1.18	9.13E-01	9.53E-01
Hexanoate	1.00	0.94	1.07	9.53E-01	9.63E-01
Ethanolamine phosphate	1.01	0.87	1.16	9.41E-01	9.63E-01
Homovanillate	1.00	0.89	1.14	9.53E-01	9.63E-01
Hypoxanthine	1.00	0.86	1.17	9.76E-01	9.76E-01

FDR, false discovery rate; NAFLD, nonalcoholic fatty liver disease

**p* < 0.05

All metabolites were log-transformed. Fold changes of metabolites from non-NAFLD to NAFLD were estimated using linear mixed-effects model with batch numbers of metabolite measurements as a random effect variable. The model was adjusted for sex, age, body mass index, smoking status (never, past, and current smokers), and alcohol-drinking status (never, past, and current drinkers).

Supplemental Table 3. Association between metabolites and CAVI values (results for all metabolites examined)

Metabolites	Coefficients	95% confidence interval		<i>p</i> -value	FDR-adjusted <i>p</i> -value
		lower	upper		
Glutamic Acid	0.19	0.12	0.26	3.45E-08*	1.07E-06*
Tyrosine	0.15	0.08	0.21	6.39E-06*	9.90E-05*
Leucine	0.16	0.09	0.24	2.65E-05*	2.74E-04*
Proline	0.13	0.06	0.20	1.31E-04*	8.92E-04*
Valine	0.13	0.06	0.20	1.73E-04*	8.92E-04*
Isoleucine	0.14	0.07	0.22	1.67E-04*	8.92E-04*
Alanine	0.10	0.04	0.17	1.17E-03*	5.20E-03*
Glycerophosphorylcholine	-0.09	-0.15	-0.04	1.47E-03*	5.70E-03*
4-Methyl-2-oxopentanoate	0.10	0.03	0.17	4.88E-03*	1.68E-02*
Tryptophan	0.08	0.02	0.15	1.49E-02*	4.62E-02*
2-Oxoisopentanoate	0.07	0.01	0.14	1.87E-02*	5.27E-02
Lactate	0.07	0.01	0.14	3.14E-02*	8.12E-02
Phenylalanine	0.06	0.00	0.12	5.52E-02	1.32E-01
Carnitine	0.06	-0.01	0.12	9.39E-02	2.08E-01
Glycerophosphate	-0.05	-0.10	0.01	1.07E-01	2.22E-01
Lysine	0.05	-0.01	0.12	1.22E-01	2.24E-01
Citrulline	0.05	-0.01	0.12	1.23E-01	2.24E-01
Malonate	-0.04	-0.11	0.02	1.64E-01	2.83E-01
Urate	0.04	-0.02	0.11	2.02E-01	3.30E-01
Pyruvate	0.03	-0.02	0.09	2.38E-01	3.69E-01
Asparagine	0.03	-0.03	0.09	3.32E-01	4.90E-01
Succinate	-0.03	-0.10	0.04	3.67E-01	5.17E-01
Serine	0.02	-0.04	0.08	4.28E-01	5.53E-01
Cysteine-glutathione disulphide -Divalent	-0.02	-0.08	0.04	4.22E-01	5.53E-01
Hippurate	-0.02	-0.08	0.04	4.79E-01	5.93E-01
Isocitrate	0.02	-0.05	0.08	5.81E-01	6.92E-01
2-Hydroxybutyrate	0.02	-0.05	0.08	6.04E-01	6.94E-01
cis-Aconitate	0.01	-0.05	0.08	6.42E-01	7.10E-01
Guanidinosuccinate	-0.01	-0.07	0.05	8.11E-01	8.67E-01
Betaine	0.004	-0.06	0.07	9.08E-01	9.38E-01
Glycine	-0.001	-0.06	0.06	9.85E-01	9.85E-01

FDR, false discovery rate

**p* < 0.05

For 31 metabolites with FDR-adjusted *p* values < 0.05 in the prior analysis, coefficients per 1 SD of log-transformed metabolites for increased CAVI values were estimated using linear mixed-effects model with batch numbers of metabolite measurements as a random effect variable. The model was adjusted for sex, age, body mass index, smoking status (never, past, and current smokers), and alcohol-drinking status (never, past, and current drinkers).

Supplemental Table 4. Association between NAFLD and metabolites after adding diabetes, hypertension, and dyslipidemia to adjustment variables (results for all metabolites)

Metabolites	Fold change	95% confidence interval		<i>p</i> -value	FDR-adjusted <i>p</i> -value
		lower	upper		
Isoleucine	1.10	1.07	1.13	5.77E-12*	4.56E-10*
Glutamic Acid	1.22	1.15	1.29	9.71E-12*	4.56E-10*
Valine	1.08	1.05	1.10	7.09E-11*	2.22E-09*
Leucine	1.08	1.05	1.10	1.68E-10*	3.94E-09*
4-Methyl-2-oxopentanoate	1.09	1.06	1.12	6.36E-08*	1.20E-06*
Tyrosine	1.07	1.04	1.10	5.75E-07*	9.01E-06*
Proline	1.09	1.05	1.13	1.99E-05*	2.68E-04*
2-Oxoisopentanoate	1.06	1.03	1.10	4.09E-05*	4.80E-04*
Alanine	1.07	1.03	1.10	8.81E-05*	9.21E-04*
Glycine	0.93	0.89	0.97	2.24E-04*	2.11E-03*
Citrulline	0.94	0.91	0.97	2.72E-04*	2.33E-03*
Serine	0.95	0.92	0.98	4.07E-04*	2.82E-03*
Cysteine-gluthathione disulphide -Divalent	0.80	0.71	0.90	3.71E-04*	2.82E-03*
2-Hydroxybutyrate	1.11	1.05	1.17	4.20E-04*	2.82E-03*
Betaine	0.93	0.90	0.97	5.44E-04*	3.01E-03*
Lactate	1.07	1.03	1.11	5.05E-04*	3.01E-03*
Isocitrate	1.07	1.03	1.11	5.35E-04*	3.01E-03*
Pyruvate	1.11	1.04	1.19	2.30E-03*	1.20E-02*
Tryptophan	1.04	1.01	1.06	3.10E-03*	1.54E-02*
Urate	1.05	1.02	1.09	4.90E-03*	2.30E-02*
Malonate	0.84	0.73	0.96	1.19E-02*	5.34E-02
Hippurate	0.73	0.57	0.94	1.38E-02*	5.89E-02
Asparagine	0.97	0.95	0.99	1.45E-02*	5.93E-02
Lysine	1.03	1.00	1.05	2.13E-02*	7.90E-02
Phenylalanine	1.03	1.00	1.05	2.03E-02*	7.90E-02
Glycerophosphate	1.09	1.01	1.17	2.18E-02*	7.90E-02
Succinate	0.96	0.93	0.99	2.34E-02*	8.16E-02
Glycerophosphorylcholine	0.83	0.70	0.98	2.46E-02*	8.27E-02
Guanidinosuccinate	0.81	0.67	0.97	2.55E-02*	8.28E-02
Choline	0.97	0.94	1.00	2.80E-02*	8.77E-02
Carnitine	1.03	1.00	1.05	4.01E-02*	1.22E-01
Threonine	0.97	0.94	1.00	4.95E-02*	1.44E-01
gamma-Butyrobetaine	0.97	0.94	1.00	5.25E-02	1.44E-01
Histidine	1.02	1.00	1.04	5.38E-02	1.44E-01
cis-Aconitate	1.04	1.00	1.08	5.20E-02	1.44E-01
Glucuronate	1.07	0.99	1.16	7.40E-02	1.93E-01
trans-Aconitate	0.89	0.77	1.02	8.44E-02	2.14E-01
N-Acetylaspartate	0.96	0.92	1.01	1.00E-01	2.42E-01
Mucate	0.96	0.92	1.01	9.94E-02	2.42E-01
Creatine	1.05	0.99	1.12	1.15E-01	2.71E-01
Sarcosine	0.90	0.78	1.03	1.33E-01	3.04E-01
proline betaine	0.88	0.74	1.05	1.46E-01	3.08E-01
2-Oxobutyrate	0.88	0.74	1.05	1.48E-01	3.08E-01
threonate	0.97	0.94	1.01	1.44E-01	3.08E-01
Citrate	0.98	0.95	1.01	1.41E-01	3.08E-01
Glutamine	1.01	0.99	1.03	1.70E-01	3.47E-01
Trigonelline	0.82	0.62	1.10	1.83E-01	3.66E-01
Trimethylamine N-oxide	0.91	0.78	1.05	1.93E-01	3.69E-01
Quinate	0.85	0.67	1.08	1.89E-01	3.69E-01

(Cont. Supplemental Table 4)

Metabolites	Fold change	95% confidence interval		<i>p</i> -value	FDR-adjusted <i>p</i> -value
		lower	upper		
Methionine	1.02	0.99	1.06	2.15E-01	4.04E-01
3-Indoxyl sulfate	1.08	0.95	1.22	2.35E-01	4.33E-01
3-Methylhistidine	1.05	0.96	1.16	2.69E-01	4.73E-01
Isethionate	0.97	0.91	1.03	2.71E-01	4.73E-01
4-Acetylbutyrate	1.09	0.94	1.26	2.70E-01	4.73E-01
Guanidinoacetate	0.98	0.94	1.02	2.85E-01	4.86E-01
Pipecolate	0.97	0.90	1.04	3.46E-01	5.80E-01
alpha-Aminoadipate	1.07	0.93	1.24	3.58E-01	5.91E-01
Arginine	0.99	0.96	1.01	3.78E-01	5.92E-01
o-Acetylcarnitine	1.05	0.94	1.17	3.90E-01	5.92E-01
Cystine	1.01	0.99	1.03	3.90E-01	5.92E-01
3-Hydroxybutyrate	0.95	0.86	1.06	3.91E-01	5.92E-01
Fumarate	1.03	0.96	1.12	3.84E-01	5.92E-01
Pelargonate	1.02	0.97	1.07	4.10E-01	6.12E-01
Creatinine	0.99	0.96	1.02	4.36E-01	6.35E-01
Aspartic acid	1.04	0.94	1.14	4.39E-01	6.35E-01
Glutarate	1.03	0.96	1.10	4.57E-01	6.51E-01
Uridine	0.99	0.97	1.01	4.88E-01	6.77E-01
Heptanoate	0.96	0.85	1.08	4.90E-01	6.77E-01
Citraconate	0.94	0.76	1.15	5.25E-01	7.15E-01
Kynurenine	1.03	0.93	1.13	5.70E-01	7.66E-01
ADMA	0.97	0.84	1.11	6.19E-01	8.11E-01
2-Oxoglutarate	0.98	0.93	1.05	6.21E-01	8.11E-01
beta-Ala	1.02	0.94	1.10	6.64E-01	8.38E-01
Ornithine	1.01	0.97	1.04	6.57E-01	8.38E-01
Cysteine S-sulfate	0.98	0.90	1.07	6.68E-01	8.38E-01
Azelate	0.99	0.93	1.05	6.84E-01	8.46E-01
5-Oxoproline	0.99	0.97	1.02	6.96E-01	8.50E-01
Hydroxyproline	1.01	0.96	1.06	7.37E-01	8.81E-01
Malate	0.99	0.95	1.03	7.41E-01	8.81E-01
2AB	0.99	0.95	1.04	7.81E-01	9.02E-01
Hypoxanthine	0.98	0.84	1.15	8.16E-01	9.02E-01
SDMA	0.98	0.86	1.12	8.14E-01	9.02E-01
Hexanoate	1.01	0.94	1.08	7.91E-01	9.02E-01
Decanoate	1.01	0.94	1.08	8.02E-01	9.02E-01
Homovanillate	0.98	0.87	1.12	7.95E-01	9.02E-01
N,N-Dimethylglycine	1.00	0.94	1.05	8.68E-01	9.08E-01
Taurine	1.00	0.98	1.03	8.32E-01	9.08E-01
Triethanolamine	0.98	0.78	1.23	8.59E-01	9.08E-01
Ethanolamine phosphate	0.99	0.85	1.14	8.64E-01	9.08E-01
Octanoate	1.01	0.89	1.14	8.79E-01	9.08E-01
Terephthalate	1.00	0.96	1.05	8.79E-01	9.08E-01
Indole-3-acetate	0.99	0.83	1.19	9.37E-01	9.57E-01
1-Methylnicotinamide	1.00	0.84	1.20	9.74E-01	9.84E-01
3-Aminoisobutyrate	1.00	0.84	1.19	9.92E-01	9.92E-01

FDR, false discovery rate; NAFLD, nonalcoholic fatty liver disease

**p* < 0.05

All metabolites were log-transformed. Fold changes of metabolites from non-NAFLD to NAFLD were estimated using linear mixed-effects model with batch numbers of metabolite measurements as a random effect variable. The model was adjusted for sex, age, body mass index, smoking status (never, past, and current smokers), alcohol-drinking status (never, past, and current drinkers), diabetes, hypertension, and dyslipidemia.

Supplemental Table 5. Association between metabolites and CAVI values after adding diabetes, hypertension, and dyslipidemia to adjustment variables (results for all metabolites examined)

Metabolites	Coefficients	95% confidence interval		<i>p</i> -value	FDR-adjusted <i>p</i> -value
		lower	upper		
Glutamic Acid	0.16	0.10	0.23	1.93E-06*	3.87E-05*
Tyrosine	0.14	0.08	0.20	1.31E-05*	1.31E-04*
Leucine	0.14	0.07	0.22	1.95E-04*	1.30E-03*
Valine	0.12	0.05	0.18	7.97E-04*	3.98E-03*
Proline	0.11	0.04	0.17	1.59E-03*	6.38E-03*
Isoleucine	0.11	0.03	0.18	5.22E-03*	1.74E-02*
Alanine	0.08	0.02	0.14	1.37E-02*	3.91E-02*
Tryptophan	0.08	0.01	0.14	1.85E-02*	4.62E-02*
4-Methyl-2-oxopentanoate	0.08	0.01	0.14	2.90E-02*	6.44E-02
2-Oxoisopentanoate	0.06	0.00	0.12	5.27E-02	1.05E-01
Lactate	0.04	-0.02	0.10	2.31E-01	4.03E-01
Citrulline	0.04	-0.03	0.10	2.42E-01	4.03E-01
Serine	0.03	-0.03	0.09	2.98E-01	4.59E-01
Pyruvate	0.02	-0.04	0.07	5.87E-01	8.29E-01
Urate	0.01	-0.05	0.08	6.64E-01	8.29E-01
Betaine	0.01	-0.05	0.08	6.62E-01	8.29E-01
Isocitrate	-0.01	-0.07	0.06	8.15E-01	8.58E-01
Glycine	0.01	-0.05	0.06	7.40E-01	8.58E-01
Cysteine-glutathione disulphide -Divalent	-0.01	-0.07	0.05	7.92E-01	8.58E-01
2-Hydroxybutyrate	0.001	-0.06	0.06	9.74E-01	9.74E-01

FDR, false discovery rate

* $p < 0.05$

For 20 metabolites with FDR-adjusted p values < 0.05 in the prior analysis, coefficients per 1 SD of log-transformed metabolites for increased CAVI values were estimated using linear mixed-effects model with batch numbers of metabolite measurements as a random effect variable. The model was adjusted for sex, age, body mass index, smoking status (never, past, and current smokers), and alcohol-drinking status (never, past, and current drinkers), diabetes, hypertension, and dyslipidemia