Introduction

Atherosclerotic cardiovascular diseases (CVDs), including coronary artery disease (CAD) and stroke, are among the leading causes of death and impairment of quality of life in patients with type 2 diabetes mellitus (T2DM)\(^1\). Therefore, the early and accurate identification of groups at high risk for CVD is important...
in the management of patients with diabetes. However, there are significant limitations in cardiovascular risk assessment based on conventional risk factors, and the underlying mechanisms cannot be fully explained by these risk factors, since CVDs are caused by complex metabolic disorders due to the interaction of many genetic and environmental factors. Thus, a novel approach to assess the comprehensive metabolic status in atherosclerosis or CVD is needed to increase their mechanistic understanding and to develop diagnostic and prognostic strategies.

Metabolomics, the study of small-molecule metabolites that are intermediates and end products of a variety of biochemical and cellular processes, is an emerging tool for research on human diseases. It allows the high-throughput identification and quantification of large numbers of metabolites across multiple pathways. Therefore, metabolomics can depict the current global metabolic fingerprinting of cells, tissues, organs, and organisms. Especially, non-targeted and unbiased metabolic profiling can be useful for both the discovery of new biomarkers and the broadening of our knowledge of disease pathophysiology.

Gas chromatography coupled with mass spectrometry (GC/MS)-based metabolome analysis has a large coverage of compounds by using derivatization that increases the volatility of the substances. Moreover, GC/MS offers high peak resolution and high reproducibility of retention times in GC, and can utilize many mass spectra libraries because of its high reproducibility of electron ionization in MS. Such characteristics of GC/MS allow a relatively easy identification of detectable peaks. Therefore, GC/MS-based metabolomics is suitable for a non-targeted approach.

In this study, we aimed to identify metabolites associated with atherosclerosis in patients with T2DM, using GC/MS-based non-targeted metabolomics optimized for multiple measurements of human blood specimens.

**Methods**

**Subjects**

The participants of this study were selected from Japanese patients with T2DM who visited Osaka University Medical Hospital between June 2014 and October 2016. Diabetes was diagnosed based on the criteria of the Japan Diabetes Society, as follows: early-morning fasting plasma glucose (PG) ≥ 126 mg/dL; 2-hour PG after 75 g glucose load ≥ 200 mg/dL; casual PG load ≥ 200 mg/dL; or anti-diabetic medication use. Screening of the potential study subjects was performed consecutively. In total, 176 non-CVD subjects who had never had a CVD event, which was defined as CAD, cerebrovascular disease, and peripheral artery disease, and 40 subjects who were survivors of CAD events were enrolled. In the present study, a CAD event was defined as acute myocardial infarction (AMI), angina pectoris (AP), or coronary revascularization treatment that included coronary intervention and coronary bypass graft. Subjects with CAD were individuals diagnosed as having AMI (n=15) or AP (n=17) and individuals who had undergone coronary revascularization treatment for myocardial ischemia (n=8).

**Ethics, Consent, and Permissions**

The study protocol was approved by the Research Ethics Committee of Osaka University Hospital (approval number 13454-6), and the study was conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all the subjects after they received a full explanation of the study.

**Clinical and Biochemical Analyses**

The following clinical data on each patient were obtained at entry: age, sex, body mass index, cardiac risk factors, prior cardiac disease, and medication. Fasting blood samples were collected and HbA1c, serum total, LDL and HDL cholesterol, serum triglyceride (TG), and serum creatinine levels were measured using standard laboratory protocols. HbA1c was determined by HPLC. LDL-C and HDL-C were measured by homogeneous assays (Sekisui Medical Co. Ltd., Tokyo, Japan) for which the accuracy had been confirmed previously. At the same time, fasting plasma used for metabolomics analysis was collected and cooled immediately in a freezer at 4°C. It was then centrifuged (3,000 x g, 10 min) and stored at −80°C within 4 hours.

The determination of hypertension (defined as systolic blood pressure (SBP) ≥ 130 mmHg; diastolic blood pressure ≥ 80 mmHg; or anti-hypertensive medication use) and dyslipidemia (defined as serum LDL-C ≥ 3.1 mmol/L (120 mg/dL); serum TG ≥ 1.7 mmol/L (150 mg/dL); HDL-C < 1.0 mmol/L (40 mg/dL); or lipid-lowering medication use) was based on the criteria of the Japan Diabetes Society. The estimated glomerular filtration rate (eGFR mL/min/1.73 m²) was calculated using an equation proposed by the Japanese Society of Nephrology.

**Assessment of Carotid IMT and FMD**

Two established markers of subclinical atherosclerosis (carotid maximal intima-media thickness (max-IMT) and flow-mediated vasodilation (FMD))
were measured as follows.

B-mode ultrasonography of the carotid artery was performed with a 7.5-MHz linear transducer. All scanning was conducted by experienced laboratory physicians using the same measuring method, in accordance with the guidelines of the Japan Society of Ultrasonics in Medicine. The thickest point of the IMT in the common carotid artery, the carotid bulb, and the internal carotid artery were measured separately, and the highest value among them was defined as max-IMT, a representative value for each individual.

FMD of the brachial artery was measured in a quiet, temperature-controlled room on the morning after at least 12 hours of fasting by using the UNEX EF 38G (UNEX Corporation, Nagoya, Japan). All measurements were performed by a single expert investigator in a manner reported previously, to minimize intra- and inter-investigator variations. To summarize, the forearm cuff was inflated to 50 mmHg above the SBP and maintained for 5 min before deflation. After deflation, the vessel diameter was measured and the maximum value was recorded. FMD was calculated as follows: 

\[ \text{FMD} (\%) = \left( \frac{\text{maximum diameter} - \text{diameter at rest}}{\text{diameter at rest}} \right) \times 100 \]

Sample Preparation for Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

All the samples were divided into 9 batches, with 20–30 samples per batch after randomization. For each batch, a series of samples was prepared and subsequent GC/MS measurements were performed.

To select the extraction method suitable for this study, the deproteinization efficiency of three preparation methods were compared, and the extraction procedure of the water:acetonitrile (MeCN) (1:4) method was adopted. (Details of the method and results of this preliminary experiment are described in Supplementary method and Supplementary Table 1). To explain this method briefly, 50 µL of plasma was mixed with 150 µL of deaerated H₂O containing 0.2 mg/mL of ribitol, which was used as an internal standard (1,200 rpm, 10 min, 4°C). Then, 800 µL of deaerated MeCN was added (1200 rpm, 10 min, 4°C) and centrifuged (16,000 x g, 3 min, 4°C). Next, 300 µL of supernatant was transferred to an Eppendorf tube. All the steps up to this point were performed in a cold room at 4°C.

The samples were then dried in a vacuum centrifuge dryer for 1 hour and lyophilized overnight. For derivatization, first, 100 µL of methoxyamine hydrochloride in pyridine (20 mg/mL) was added to the samples, and the mixture was incubated (1,200 rpm, 90 min, 30°C). A second derivatizing agent, N-methyl-N-trimethylsilyl-trifluoroacetamide, was then added and the mixture was incubated (1,200 rpm, 30 min, 37°C). After centrifugation (16,000 x g, 3 min), 100 µL of the derivatized samples was transferred to glass vials.

GC/MS Analysis and Data Processing

The metabolic profiling analysis was conducted on a Shimadzu TQ8040 GC system (Shimadzu Corporation, Kyoto, Japan) that was connected to a mass spectrometer. The samples (1 µL each) were injected into the GC/MS system in split mode (split ratio 1:25). An InertCap 5MS/NP capillary column (GL Sciences Inc., Tokyo, Japan) was used. The injection temperature was 270°C. The helium gas flow rate through the column was 1.12 mL/min. The column temperature was set to 80°C for 2 min and then raised to 330°C for 12 min. The temperature of the transfer interface and ion source was set to 310°C and 280°C, respectively. The selected mass range was set to 85–500 m/z with electron impact ionization (70 eV).

All 216 plasma samples were analyzed after randomizing the sample sequence over multiple batches. A quality control (QC) sample, n-alkane mix C9-C40 (GL Sciences Inc.) containing decafluorotriphenylmethane phosphine (DFTPP) (Sigma-Aldrich, Tokyo, Japan) were injected after every 5 study samples to monitor the stability of the analytical system. The QC samples were generated by mixing the same volume of plasma from 20 healthy subjects.

It was confirmed that overall MS sensitivity was highly stable, based on the relative intensity of each fragment of DFTPP (Supplementary Fig. 1). In addition, the residual standard deviation (RSD) of intensity of ribitol (internal control) in all study samples and QC samples was 10.0%, indicating high intra- and inter-day stability of the overall GC/MS measurement, including plasma extraction and derivatization steps.

The obtained GC/MS data were converted to an Analysis Base File (ABF) format using an ABF converter (https://www.reifycs.com/AbfConverter/index.html). Feature detection, spectra deconvolution, metabolite identification, and peak alignment were performed using MS-DIAL software ver. 2.72. This software is freely available from http://prime.psc.riken.jp/Metabolomics_Software/MS-DIAL/index.html. Annotations in MS-DIAL were performed by matching the obtained RI and MS spectra with GL-Science DB (https://prime.psc.riken.jp/Metabolomics_Software/MS-DIAL/index.html), which is a freely available library. Each metabolite was calibrated using the LOWESS/Spline correction curve based on the QC values, after the substances for which the RSD of QC samples was above 40% were excluded from...
Table 1. Clinical characteristics of study subjects

|                                | Non-CVD subjects | CAD subjects | p-value |
|--------------------------------|------------------|--------------|---------|
| Number of subjects             | 176              | 40           |         |
| Age (years)                    | 58.4 ± 12.3      | 66.5 ± 5.4   | <0.001  |
| Male gender (n, %)             | 94 (53.4)        | 30 (75.0)    | 0.013   |
| BMI (kg/m²)                    | 27.7 ± 5.9       | 26.4 ± 4.6   | 0.212   |
| eGFR (ml/min/1.73 m²)          | 77.1 ± 24.5      | 59.1 ± 18.5  | <0.001  |
| Diabetes duration (years)      | 11.4 ± 8.9, n=174| 18.4 ± 10.9, n=39| <0.001 |
| HbA1c (%)                      | 9.1 ± 1.8        | 8.3 ± 1.5, n=39| 0.006   |
| FPG (mg/dl)                    | 154 ± 50, n=173  | 145 ± 41, n=39| 0.253   |
| Smoking history (n, %)         | 82 (48.6), n=175 | 23 (62.2), n=37| 0.150   |
| Hypertension (n, %)            | 96 (54.5)        | 39 (97.5)    | <0.001  |
| Systolic BP (mmHg)             | 125 ± 18         | 121 ± 15, n=38| 0.189   |
| Dyslipidemia (n, %)            | 106 (60.2)       | 37 (92.5)    | <0.001  |
| Total cholesterol (mg/dl)      | 202 ± 49         | 166 ± 39     | <0.001  |
| HDL cholesterol (mg/dl)        | 47.7 ± 13.2      | 49.7 ± 17.8  | 0.407   |
| LDL cholesterol (mg/dl)        | 119 ± 39         | 87 ± 24      | <0.001  |
| Triglyceride (mg/dl)           | 180 ± 140        | 173 ± 117    | 0.761   |
| FMD (%)                        | 5.96 ± 2.56, n=154| 6.16 ± 2.54, n=23| 0.721   |
| Carotid max-IMT (mm)           | 1.72 ± 0.67, n=165| 2.27 ± 0.90, n=35| <0.001  |
| Medication use                 |                  |              |         |
| Diabetes (n, %)                | 146 (83.0)       | 37 (92.5)    | 0.149   |
| Hypertension (n, %)            | 87 (49.4)        | 39 (97.5)    | <0.001  |
| Dyslipidemia (n, %)            | 68 (38.6)        | 36 (90.0)    | <0.001  |
| Antiplatelet agent (n, %)      | 15 (8.5)         | 38 (95.0)    | <0.001  |

Data are presented as mean ± standard deviation or number with percentage in parentheses. Means and Categorical variables were compared using unpaired t-test and Pearson’s chi-squared test respectively between non-CAD and CAD subjects. Bold font indicates statistically significant (p<0.05) difference.

Results

Statistical Analysis

Clinical data are reported as the means ± standard deviation for continuous variables and percentages for dichotomous variables. Means were compared using unpaired t-tests. Categorical variables were compared using Pearson’s chi-squared test. Metabolome data are shown as the median and interquartile range.

The identification of metabolites associated with atherosclerosis was performed using a two-step approach. The first step was the analysis of the associations between each metabolite and subclinical atherosclerotic markers (i.e., FMD and max-IMT) in non-CVD subjects by using Spearman’s correlation. After identifying the metabolites associated with both the atherosclerotic markers, the second step of analysis was performed to investigate the association between these metabolites and the history of CAD by using the Mann–Whitney U test in all subjects. Furthermore, the association between the metabolites detected and CAD after adjusting for clinical risk factors was evaluated by matched case–control studies with propensity scores (PS). Matching variables were age, gender, diabetes duration, eGFR, HbA1c, smoking history, hypertension, hyperlipidemia, and use of medication for T2DM, hypertension, and hyperlipidemia (1:n matching). The comparison was made between a matched CAD case and a control for each metabolite by general linear mixed-effect model analysis. In this case–control analysis, log-transformed values were used for metabolome data.

For all tests, a p-value <0.05 was considered statistically significant. These statistical analyses were performed in R version 3.4.3 (The R Foundation for Statistical Computing, www.R-project.org) and SPSS version 22 (SPSS Inc., Chicago, IL, USA).

Further analysis. As a result of these processes, the intra- and inter-day precision of the quantification of each metabolite used in this analysis was secured within a certain range; in addition, the variability of quantification of each metabolite was corrected.

Patient Characteristics

Patient characteristics of the non-CVD subjects...
Metabolites Related to Atherosclerosis

Table 2. List of metabolites significantly associated with either max-IMT or FMD in non-CVD subjects

| Metabolite             | FMD (n = 154) | max-IMT (n = 165) |
|------------------------|---------------|-------------------|
|                        | FMD (n = 154) | max-IMT (n = 165) |
|                        | p-value       | p-value           |
| 1,5-Anhydro glucitol   | -0.051        | 0.526             | 0.179 | 0.021 |
| 3-Aminoisobutyric acid | -0.173        | **0.032**         | 0.133 | 0.088 |
| Galactose + Glucose    | -0.162        | **0.045**         | 0.061 | 0.438 |
| Gluconic acid          | -0.202        | **0.012**         | 0.095 | 0.224 |
| Glucose                | -0.168        | **0.037**         | 0.090 | 0.249 |
| Glucuronic acid        | -0.166        | **0.040**         | 0.081 | 0.299 |
| Indoxyl sulfate        | -0.225        | **0.005**         | 0.228 | **0.003** |
| Inositol               | -0.163        | **0.044**         | 0.299 | **9.5 × 10^{-5}** |
| Mannose                | -0.227        | **0.005**         | -0.002 | 0.980 |
| Meso-erythritol        | -0.145        | 0.072             | 0.200 | **0.010** |
| O-Phosphoethanolamine  | -0.188        | **0.020**         | 0.000 | 0.998 |
| Pyroglutamic acid      | -0.083        | 0.305             | 0.241 | **0.002** |
| Urea                   | -0.129        | 0.112             | 0.260 | **0.001** |

Spearman rank correlation coefficient was evaluated to detect the metabolites associated with the FMD or max-IMT. Bold font indicates statistically significant (p < 0.05) difference.

Table 3. Univariate association of plasma levels of indoxyl sulfate and inositol with CAD

| Metabolite  | Non-CVD subjects (n = 176) | CAD subjects (n = 40) | p-value |
|-------------|-----------------------------|-----------------------|---------|
| Indoxyl sulfate | 1028 (663.4-1733)     | 2016 (1193-3427)   | 2.8 × 10^{-5} |
| Inositol    | 10210 (8360-12620)   | 12830 (10590-16250) | **2.6 × 10^{-4}** |

Data are presented as median with interquartile range. Mann-Whitney U test was performed to detect the metabolites significantly (p < 0.05) associated with the onset of CAD. Bold font indicates statistically significant (p < 0.05) difference.

and subjects with CAD are shown in Table 1. Mean age and diabetes duration were significantly higher, and HbA1c levels, eGFR, total cholesterol, and LDL-C were significantly lower in subjects with CAD than in non-CVD subjects. Frequencies of male gender, hypertension, and dyslipidemia were higher in subjects with CAD. In terms of medication use, pharmacological therapy for dyslipidemia and hypertension was performed more often in patients with CAD than in non-CVD subjects.

A total of 65 annotated metabolites and 84 unknown metabolites were detected from plasma samples after excluding the metabolites for which the RSD of QC samples was more than 40% (Supplementary Table 2).

Metabolites Associated with Max-IMT and FMD (Univariate Analysis)

First, biomarker candidates for atherosclerosis were screened by analyzing the association of these 149 metabolites with max-IMT and FMD in the non-CVD subjects.

Metabolites significantly associated with either max-IMT or FMD are shown in Table 2 (for annotated metabolites) and Supplementary Table 3 (for unknown metabolites). Nine of 65 annotated metabolites were significantly associated with max-IMT and six were significantly associated with FMD. Among them, only two substances, indoxyl sulfate and inositol, were significantly associated with both max-IMT and FMD: plasma levels of indoxyl sulfate and inositol were positively associated with max-IMT and negatively associated with FMD. For unknown metabolites, 5 of 84 unknown metabolites were significantly associated with both max-IMT and FMD in non-parametric univariate analysis.

Associations between Candidate Metabolites and CAD

To further investigate whether these selected metabolites are associated with CAD, their plasma levels were compared between non-CVD subjects and subjects with a history of CAD. The Mann–Whitney U test showed that plasma levels of indoxyl sulfate and inositol were both significantly higher in the subjects with CAD (Table 3). Next, based on the PS, 77 of
177 non-CVD subjects were matched with 31 of 40 subjects with CAD (the clinical characteristics of matched subjects with and without CAD are shown in Supplementary Table 4). Although inositol remained associated with CAD even after matching for traditional risk factors for atherosclerosis, between-group differences in plasma levels of indoxyl sulfate did not reach the statistical significance after adjusting for traditional risk factors (Table 4). Multiple logistic regression analyses in whole subjects adjusted for age, gender, and HbA1c revealed similar results: a high inositol level was significantly related to CAD (odds ratio [OR] per 1SD 1.37 [1.05–1.79], p = 0.022) but a high indoxyl sulfate level was not (OR per 1SD 1.25 [0.93–1.68], p = 0.134). Among the five unknown metabolites significantly associated with both atherosclerotic markers, only one metabolite (e.g., Unknown #29) was also significantly associated with CAD in univariate analysis (Supplementary Table 2). However, such an association was not demonstrated in the PS-matched population (data not shown).

### Discussion

We performed a comprehensive metabolome profiling of plasma in patients with T2DM to explore the metabolites associated with atherosclerosis, and found that plasma levels of several metabolites, including inositol and indoxyl sulfate, were associated with carotid max-IMT and/or FMD in these patients. Furthermore, among them, plasma levels of inositol and indoxyl sulfate were significantly higher in subjects with CAD than in those without an apparent history of CVD.

In the current study, we used GC/MS to analyze the metabolome profiling of plasma samples of 216 patients with T2DM, as GC/MS is a highly sensitive and high-throughput analytical platform, and thus is a useful tool for the non-targeted analysis of various samples. However, to maintain high reproducibility in non-targeted metabolomics, the establishment of an optimized protocol is critical. As blood samples contain high molecular weight species, such as proteins and lipids, that could impair the sensitivity of measurement, the step to remove such impeders is very important, especially in large-scale metabolomic profiling. Therefore, in the extraction step, we used MeCN for deproteinization, as our preliminary experiment showed that MeCN was highly efficient in protein removal compared with other organic solvents. Moreover, the extraction at 4°C may also contribute to preventing the contamination of TGs, which could also lower the sensitivity of the measurement. Indeed, our preliminary experiment revealed that protein and TGs were removed effectively by using MeCN at 4°C in comparison with that at room temperature (data not shown). Moreover, according to the relative intensity of ribitol and each fragment ratio of DFTPP, the high stability of overall GC/MS measurements was maintained through all the 216 samples, suggesting that the metabolome profile acquired in this study was highly reliable.

It is difficult to explore the association between a biomarker candidate and CVD in the cross-sectional study, as the association may be influenced by the changes caused by treatment for the secondary prevention of CVD. Therefore, in the present cross-sectional study, different atherosclerosis-related outcomes (i.e., carotid max-IMT, FMD, and a history of CAD) were prespecified to detect the metabolites associated with atherosclerosis. In the first step, candidates were screened by assessing associations of metabolites with the carotid max-IMT and FMD in the non-CVD subjects. Carotid IMT has been established as a simple and useful marker of subclinical changes in the vascular structure, an index located somewhere between risk factors and “hard” clinical end point events, such as AMI and stroke. Carotid IMT has indeed been shown to be a predictor of CVD. In contrast, FMD is a marker of early atherosclerosis causing endothelial dysfunction and is also known as a predic-

### Table 4. Association of plasma levels of indoxyl sulfate and inositol between subjects with and without CAD matched with traditional risk factors for atherosclerosis

| Metabolite   | Non-CVD subjects (n = 77) | CAD subjects (n = 31) | p-value |
|--------------|--------------------------|-----------------------|---------|
| Indoxyl sulfate | 1359 (942.1-2063)         | 1529 (941.0-3428)     | 0.21    |
| Inositol     | 11150 (8914-14030)        | 12650 (10390-14970)   | 0.04    |

Data are presented as median with interquartile range. Matching variables were age, gender, diabetes duration, eGFR, HbA1c, smoking history, hypertension, hyperlipidemia, and medication use of diabetes, hypertension, and hyperlipidemia (1:n propensity score matching). The comparison was made between matched CAD and non-CAD subjects for each metabolite by general linear mixed effect model analysis. Log-transformed values were used for metabolome data in this analysis. Bold font indicates statistically significant (p < 0.05) difference.
tor of CVD. In the second step, the association between the metabolites that directly associated with both carotid IMT and FMD and a history of CAD was evaluated. Thus, to explore the factors related to both the progressive process of atherosclerosis and the consequent onset of CAD, we first used subclinical markers to screen potential metabolites and then evaluated whether the metabolites selected were associated with a history of CAD. The major limitation of this approach is that the metabolite associated with the onset of CAD but not with max-IMT and/or FMD is overlooked. However, the metabolites associated with both a history of CAD and subclinical markers could be promising candidates related through multiple stages of the progression of atherosclerosis.

As a result, two (i.e., indoxyl sulfate and inositol) of 65 metabolites annotated by non-targeted GC/MS analysis were associated with three different atherosclerotic outcomes. Notably, there were significant associations of inositol with CAD, even after adjustments for multiple clinical covariates. This finding suggests that this substance could be a novel biomarker of atherosclerotic disease and related to the pathogenesis of atherosclerosis, independent of the major cardiovascular risk factors. However, no other papers have reported the association between blood inositol levels and atherosclerosis, except for Reddivari L et al., who reported that the blood inositol level was higher in patients with T2DM with a history of ischemic stroke than in healthy controls. Furthermore, there has been no report that indicates the mechanisms of association between plasma levels of inositol and atherosclerosis or CAD. It is also unclear whether inositol is involved directly in the atherosclerotic process, although it is well known that an intracellular deficiency of myo-inositol could play an important role in the development and progression of diabetic microvascular complications. Therefore, further studies are needed to clarify this point.

Indoxyl sulfate is also a promising biomarker candidate, according to our result. This substance is a metabolite of dietary protein or tryptophan, and is clinically known as a circulating uremic toxin. Numerous clinical studies have shown that this molecule may contribute to CVD in subjects with chronic kidney disease (CKD). For example, Lin CJ et al. had demonstrated previously that indoxyl sulfate predicts CVD in patients with CKD. Moreover, previous studies revealed that this substance directly accelerates the progression of atherosclerosis by several mechanisms, such as the inhibition of endothelial function and an increase in smooth muscle cell proliferation. Additionally, Sato et al. revealed that a higher concentration of the plasma indoxyl sulfate level was associated with increased carotid IMT in chronic patients with CAD with preserved renal function, which is consistent with the results of our study. These findings suggest that an elevation in plasma indoxyl sulfate levels could accelerate atherosclerosis not only in patients with severe renal dysfunction but also in those with early nephropathy or normal renal function. Indeed, loss of renal function is not the sole reason for high serum concentrations of uremic toxins. There is increasing interest in the gut microbiota as a relevant source of uremic toxins. Indole is produced by intestinal bacteria as a degradation product of tryptophan and is subsequently absorbed and metabolized in the liver to indoxyl sulfate. As diabetes alters the gut microenvironment profoundly and is associated with a distinct gut microbial composition and metabolism, it could be also related to the elevation of plasma indoxyl sulfate levels.

In terms of unknown metabolites, five of 84 were associated significantly with both the subclinical atherosclerotic markers. Among them, one substance was associated with CAD in the univariate analysis. Utilizing the information such as retention time and MS spectrum, these unknown candidate substances might be identified in the future. These investigations may also contribute to the elucidation of the pathophysiology of CAD.

Through the pilot analysis of serum of 55 subjects with T2DM using GC/MS, we previously reported seven candidates as metabolites that were associated with the onset of CAD. Among them, two metabolites (i.e., hypoxanthine and nonanoic acid) were also assessed in the current GC/MS analysis. Consistent with our previous study, the present study also showed that plasma levels of hypoxanthine were decreased significantly in subjects with CAD in univariate analysis, while this association had disappeared after adjusting for conventional coronary risk factors (data not shown). On the other hand, plasma levels of nonanoic acid were not associated with CAD in the present study. This inconsistency may be due to differences in the research design. Our previous report evaluated the association between the baseline levels of each metabolite and the future onset of CAD, based on cohort data. On the other hand, the current study evaluated the association of each metabolite with the past onset of CAD. Thus, the association between nonanoic acid and CAD could have been masked by the treatment for the secondary prevention of CAD in the current study. In any case, as the sample size in either study was insufficient to draw conclusions, further large-scale investigations are needed to verify the results.

In addition to the limitations described above,
several limitations of our study should be discussed. First, a history of CAD was used as an atherosclerotic outcome. The metabolic status of patients who had suffered from CAD could be influenced by various factors that changed after the onset of CAD, such as secondary prevention treatment. To minimize this disadvantage, the analysis was adjusted for major conventional risk factors. Nonetheless, we could not exclude the possibility that our results were influenced by other clinical background factors. Second, from this cross-sectional study, no conclusion could be drawn as to whether there are causal relationships between the metabolites detected and atherosclerosis. Future prospective cohort studies and experimental studies using in vivo or in vitro models will be necessary to elucidate this point. Third, we cannot present reference intervals for the metabolites measured in this study, as we did not evaluate the plasma metabolome of a general population on a large scale. Although the sample population was small, a pilot study revealed that the levels of inositol were significantly higher in non-CVD subjects with diabetes (median [inter-quartile range]) (10210 [8360–12620] vs. 9372 [7412–10670]) than in healthy subjects (n=20, mean age 34.8±6.3 years, male gender 60%). There was no significant difference in levels of indoxyl sulfate (1028 [663.4–1733] vs. 1117 [682.2–1771]). Finally, although GC/MS analysis can measure diverse classes of compounds sensitively because of its high chromatographic resolution, this approach cannot fully cover the entire metabolome of a biological sample. A combination with other MS analysis would enable the investigation of metabolome profiles with a broader coverage. Notwithstanding these limitations, our study indicates that the metabolites detected could be novel factors in association with atherosclerosis in patients with T2DM.

In conclusion, we identified novel biomarker candidates for atherosclerotic disease in Japanese patients with T2DM, using GC/MS-based non-targeted metabolomics optimized for multiple measurements of blood samples.

Declarations

Ethics Approval and Consent to Participate
The study protocol was approved by the Research Ethics Committee of Osaka University Graduate School of Medicine and the study was conducted in accordance with the principles of the Helsinki Declaration. Written informed consent was obtained from all the subjects after they received a full explanation of the study.

Consent for Publication
Not applicable.

Competing Interests
None.

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Authors’ Contributions
The authors meet the criteria for authorship recommended by the International Committee of Medical Journal Editors and take full responsibility for all contents of the manuscript and editorial decisions. All authors contributed to the study design and were involved at all stages of manuscript development. KO and NK drafted the manuscript. All authors were involved in the analysis and interpretation of data, reviewed/edited the manuscript, and approved the final manuscript. NK was the principal guarantor of this work, has full access to all the data, and takes responsibility for the integrity of the data and accuracy of data analysis.

Availability of Data and Materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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References
1) Eckel RH, Kahn R, Robertson RM, Rizza RA. Preventing cardiovascular disease and diabetes: a call to action from the American Diabetes Association and the American Heart Association. Circulation, 2006; 113: 2943-2946
2) Stephens JW, Ambler G, Vallance P, Betteridge DJ, Humphries SE, Hurel SJ. Cardiovascular risk and diabetes. Are the methods of risk prediction satisfactory? Eur J Cardiovasc Prev Rehabil, 2004; 11: 521-528
3) Simmons RK, Coleman RL, Price HC, Holman RR, Khaw KT, Wareham NJ, Griffin SJ. Performance of the UK Prospective Diabetes Study risk engine and the Framingham risk equations in estimating cardiovascular disease in the EPIC- Norfolk Cohort. Diabetes Care, 2009; 32: 708-713
4) van Dieren S, Peelen LM, Nöthlings U, van der Schouw YT, Rutten GE, Spijkerman AM, van der A DL, Sluijk D, Boeving H, Moons KG, Beulens JW. External validation of the UK Prospective Diabetes Study (UKPDS) risk engine in patients with type 2 diabetes. Diabetologia, 2011; 54: 264-270
5) Haneda M, Noda M, Origasa H, Noto H, Yabe D, Fujita Y, Goto A, Kondo T, Araki E. Japanese Clinical Practice Guideline for Diabetes 2016. J Diabetes Investig, 2018; 9: 1-45
6) Miida T, Nishimura K, Hirayama S, Miyamoto Y, Nakamura M, Masuda D, Yamashita S, Ushiyama M, Komori T, Fujita N, Yokoyama S, Teramoto T. Homogeneous Assays for LDL-C and HDL-C are Reliable in Both the Postprandial and Fasting State. J Atheroscler Thromb, 2017; 24: 583-599
7) Japan Diabetes Society, Treatment Guide for Diabetes Editorial Committee: Diabetes mellitus treatment guideline based on scientific ground (revised edition 2). Volume 1. Edited by Japan Diabetes Society. Tokyo, Japan: Nankodo; 2007: 257-272
8) Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K, Nitta K, Yamagata K, Tomino Y, Yokoyama H, Hishida A. Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis, 2009; 53: 982-992
9) Terminology and Diagnostic Criteria Committee JSoUIM: Standard method for ultrasound evaluation of carotid artery lesions. J Med Ultrason, 2009; 36: 219-226
10) Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creger MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J a d Vogel R. Guidelined for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of International Brachial Artery Reactivity Task Force. J Am Coll Carotid, 2002; 39: 257-265
11) Tomiyama H, Higashi Y, Takase B, Node K, Sata M, Inoue T, Ishibashi Y, Ueda S, Shimada K and Yamashina A. Relationships among hyperuricemia, metabolic syndrome, and endothelial function. Am J Hypertens, 2011; 24: 770-774
12) Hiroyo Ninomiya, Naoto Katakami, Ihoko Sato, Saeko Osawa, Yuichi Yamamoto, Mitsuyoshi Takahara, Dan Kawamori, Taka-aki Matsuoka, Ichiro Shimomura. Association between subclinical atherosclerosis markers and the level of accumulated advanced glycation end-products in the skin of patients with diabetes. J Atheroscler Thromb, 2018; doi: 10.5551/jat.44859
13) Tsugawa H, Caijk T, Kind T, Ma Y, Higgins B, Ikeda K, Kanazawa M, Vandersd MOVYs J, Fiehn O, Arita M. MS-DIAL: data-independent MS/MS deconvolution for comprehensive metabolome analysis. Nat Methods, 2015; 12: 523-526
14) Tsugawa H, Kanazawa M, Ogiwara A, Arita M. MRM-PROBS suite for metabolomics using large-scale MRM assays. Bioinformatics, 2014; 30: 2379-2380
15) Dunn WB, Broadhurst D, Begley P, Zelena E, Francis McIntyre S, Anderson N, Brown M, Knowles JD, Halsell A, Haselden JN, Nicholls AW, Wilson JD, Kell DB, Goodacre R. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. Nat Protoc, 2011; 30: 1060-1083
16) Blanchard J. Evaluation of the relative efficacy of various techniques for deproteinizing plasma samples prior to high-performance liquid chromatographic analysis. J Chromatogr, 1981; 226: 455-460
17) Polson C, Sarkar P, Incledon B, Raguvan V, Grant R. Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci, 2003; 785: 263-275
18) Katakami N, Mita T, Gosho M, Takahara M, Irie Y, Yasuda T, Matsuoka TA, Osono T, Watada H, Shimomura I. Clinical utility of carotid ultrasonography in the prediction of cardiovascular events in patients with diabetes - a combined analysis of data obtained in five longitudinal studies. J Atheroscler Thromb, 2018; doi: 10.5551/jat.43141
19) O’Leary DH, Polak JF, Krommal RA, Manolio TA, Burke GL, Wolfson SK Jr. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. N Engl J Med, 1999; 340: 14-22
20) Lorenz MW, Markus HS, Bots ML, Rosvall M, Sitzer M. Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis. Circulation, 2007; 115: 459-467
21) Schachinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation, 2000; 101: 1899-1906
22) Suwaidi JA, Hamasaki S, Higano RA, Nishimura RA, Holmes DR Jr., Lerman A. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. Circulation, 2000; 101: 948-954
23) Reddiri V, Sapkota BR, Rudraraju A, Liang Y, Aston C, Sidorov E, · Vanamala JKP, Sanghera DK. Metabolic signature of diabetes with cardiovascular disease: a pilot investigation. Metabolomics, 2017; 13: 154
24) Lin CJ, Liu HL, Pan CF, Chuang CK, Jayakumar T, Wang TJ, Chen HH, Wu CJ. Indoxyl sulfate predicts cardiovascular disease and renal function deterioration in advanced chronic kidney disease. Arch Med Res, 2012; 43: 451-456
25) Hung SC, Kuo KL, Wu CC, Tarn DC. Indoxyl Sulfate: A Novel Cardiovascular Risk Factor in Chronic Kidney Disease. J Am Heart Assoc, 2017; 6: e005022
26) Vanholder R, Schepers E, Pletinck A, Nagler EV, Glorieux G. The Uremic Toxicity of Indoxyl Sulfate and p-Cresyl Sulfate: A Systematic Review. J Am Soc Nephrol, 2014; 25: 1897-1907
27) Sato B, Yoshikawa D, Ishii H, Kikuchi R, Arima T, Takeshita K, Inoue Y, Suzuki S, Tanaka M, Kumasai G, Matsumoto M, Hayashi M, Ando H, Amano T, Matsubara T, Niwa T, Murohara T. Indoxyl sulfate, a uremic toxin, and carotid intima-media thickness in patients with coronary artery disease. Int J Cardiol, 2013; 163: 214-216
28) Ramezani A, Massy ZA, Meijers B, Evenepoel P, Van
holder R, Raj DS. Role of the Gut Microbiome in Uremia: A Potential Therapeutic Target. Am J Kidney Dis, 2016; 67: 483-489

29) Sabatino A, Regolisti G, Brusasco I, Cabassi A, Morabito S, Fiaccadori E. Alterations of intestinal barrier and microbiota in chronic kidney disease. Nephrol Dial Transplant, 2015; 30: 924-933

30) Sabatino A, Regolisti G, Cosola C, Gesualdo I, Fiaccadori E. Intestinal Microbiota in Type 2 Diabetes and Chronic Kidney Disease. Curr Diab Rep, 2017; 17: 16

31) Sato J, Kanazawa A, Ikeda F, Yoshihara T, Goto H, Abe H, Komiya K, Kawaguchi M, Shimizu T, Ogihara T, Tamura Y, Sakurai Y, Yamamoto R, Mita T, Fujitani Y, Fukuda H, Nomoto K, Takahashi T, Asahara T, Hirose T, Nagata S, Yamashiro Y, Watada H. Gut Dysbiosis and Detection of “Live Gut Bacteria” in Blood of Japanese Patients With Type 2 Diabetes. Diabetes Care, 2014; 37: 2343-2350

32) Omori K, Katakami N, Yamamoto Y, Ninomiya H, Takahara M, Matsuoka TA, Bamba T, Fukusaki E, Shimomura I. Identification of Metabolites Associated with Onset of CAD in Diabetic Patients Using CE-MS Analysis: A Pilot Study. J Atheroscler Thromb, 2019; 26: 233-245
Supplementary Method

Protocol to evaluate the deproteinization efficacy of three different extraction methods.

Extraction procedure

Sterile human plasma was obtained from Rockland Immunochemicals Inc. (Gilbertsville, PA, USA).

Extraction procedure of Methanol:Water:Chloroform (5:1:2) method was as follows: First, 400 µL of the above mixed solvent was added to 50 µL of plasma and shaken (1200 rpm, 30 min, 4°C). Next, 400 µL of water was added and mixed. After further centrifugation (16,000 g, 3 min, 4°C), 600 µL of the supernatant was used as an extract.

Extraction procedure of Water:Acetonitrile (1:4) method and Water:Methanol (1:4) method were as follows: First, 150 µL of water was added to 50 µL of plasma and shaken (1,200 rpm, 10 min, 4°C). Subsequently, 800 µL of the organic solvent was added and mixed (1,200 rpm, 30 min, 4°C). After centrifugation (16,000 g, 3 min, 4°C), 800 µL of the supernatant was collected and used as an extract.

Protein quantification procedure

To evaluate the deproteinization efficiency of each extraction method, protein quantification was performed. Takara BCA Protein Kit was purchased from Takara Bio Inc. (Shiga, Japan) and quantification was performed in accordance with the standard protocol of the kit. Briefly, 200 µL of working solution was added to 10 µL of the extraction solution and reacted (30 min, 37°C). Thereafter, the absorbance at 562 nm was measured using a spectrophotometer. Water dilution sample (50 µL plasma + 950 µL water) was used as Control. Quantitative values were calculated using a calibration curve prepared from BSA standard solution, and protein residuals were indicated as relative values to Control.

Supplementary Table 1. Protein residual ratio of each extraction method (n=7)

| Extraction solvent (ratio) | Protein residual ratio (%) |
|---------------------------|---------------------------|
| MeOH:H2O:CHCl3 (5:1:2)    | 15.5±2.2                  |
| H2O:MeCN (1:4)            | 0.5±0.3                   |
| H2O:MeOH (1:4)            | 3.3±0.9                   |

Supplementary Fig. 1. Relative intensity of each fragment of DFTPP

MS sensitivity was monitored using DFTPP, which was injected after every 5 study sample measurements. Relative intensity of each fragment of DFTPP was stable through the measurement of all of the 240 samples.
 Supplementary Table 2. List of all metabolites detected by GC/MS analysis in subjects in each group, with and without CAD

| Metabolite                        | Non-CVD subjects (n = 176) | CAD subjects (n = 40) | p-value |
|-----------------------------------|-----------------------------|-----------------------|---------|
|                                   | Median                      | Interquartile range   |         |
|                                   | 25th | 75th                 | median  | Interquartile range | 25th | 75th |
| 1,5-Anhydro glucitol              | 7158 | 4053 13323            | 12002   | 6841 18446          | 0.002 |
| 1-Hexadecanol                     | 31394 | 24375 39660          | 32760   | 24993 37261          | 0.922 |
| 2-Aminobutyric acid               | 14092 | 11299 18188          | 12014   | 8518 17765          | 0.107 |
| 2-Aminoethanol                    | 3452  | 2907 4146            | 3561    | 3035 4374           | 0.287 |
| 2-Hydroxybutyrate                 | 20641 | 16545 26894          | 16199   | 11055 24369         | 0.014 |
| 2-Hydroxypridine                  | 3953  | 3587 4369            | 3976    | 3448 4531           | 0.955 |
| 3-Amino isobutyric acid           | 1664  | 962 2343             | 1882    | 977 2559           | 0.498 |
| Alamine TMS                       | 118231 | 96391 164060         | 121565  | 91122 183365        | 0.610 |
| Alamine 3TMS                      | 6780  | 5328 8778            | 6677    | 5141 7727           | 0.461 |
| Allose + mannose                  | 2408  | 1946 3033            | 2148    | 1876 2907           | 0.237 |
| Asparagine                        | 2588  | 2122 3122            | 2465    | 2240 2824           | 0.374 |
| Cholesterol                       | 41933 | 36961 48946          | 38373   | 34574 45713         | 0.046 |
| Creatinine                        | 3300  | 2532 4352            | 3752    | 2901 5369           | 0.014 |
| Fructose                          | 3206  | 2477 4188            | 3673    | 2253 4474           | 0.397 |
| Galactose + glucose               | 534444 | 438142 678112        | 525087  | 417030 624005       | 0.256 |
| Gluconic acid                     | 646   | 476 965              | 692     | 454 910            | 0.829 |
| Glucose                           | 2918639 | 2393141 3689212      | 2828617 | 2344971 3434026     | 0.293 |
| Glucuronate                       | 672   | 423 953              | 721     | 565 1099           | 0.113 |
| Glutamic acid                     | 10750 | 7405 15581           | 9246    | 7282 12681          | 0.095 |
| Glutamine                         | 69994 | 52267 100177         | 69333   | 55161 118601        | 0.308 |
| Glyceric acid                     | 2316  | 1859 2736            | 2322    | 1678 2909           | 0.937 |
| Glycine                           | 87860 | 74650 104061         | 85690   | 74287 99249         | 0.707 |
| Glycolic acid                     | 5181  | 4308 6018            | 4695    | 4150 5478           | 0.065 |
| Histidine                         | 6907  | 5436 10129           | 6696    | 5203 11171          | 0.971 |
| Hydroxyproline                    | 2450  | 1966 3094            | 2311    | 1832 2754           | 0.130 |
| Hypoxanthine                      | 1222  | 628 2357            | 834     | 394 1752           | 0.021 |
| Indoxyl sulfate                   | 1028  | 663 1733            | 2016    | 1193 3427           | 0.000 |
| Inositol                          | 10213 | 8360 12622           | 12829   | 10586 16250         | 0.000 |
| Isoiceric acid + citric acid      | 13581 | 11608 16541          | 14562   | 11640 18118         | 0.313 |
| Isoleucine 1TMS                   | 3123  | 2178 4908            | 3629    | 2760 5469           | 0.143 |
| Isoleucine 2TMS                   | 46082 | 35921 58987          | 46033   | 36739 60532         | 0.942 |
| Lactic acid                       | 448981 | 351059 559214        | 433807  | 331303 565760       | 0.431 |
| Lauric acid                       | 2622  | 2151 3403            | 2729    | 2114 3328           | 0.909 |
| Leucine 1TMS                      | 6355  | 4240 9124            | 6359    | 4296 8859           | 0.958 |
| Leucine 2TMS                      | 93011 | 69956 121461          | 84292   | 72574 116639         | 0.297 |
| Lysine                            | 29769 | 26677 34773           | 26948   | 22684 34303         | 0.052 |
| Mannitol                          | 1079  | 675 1777             | 2374    | 1205 6454           | 0.000 |
| Mannose                           | 26807 | 21341 33005           | 28267   | 23095 31379         | 0.884 |
| Meso erythritol                   | 2968  | 2074 5984            | 4197    | 2545 11376          | 0.023 |
| Methionine                        | 5863  | 4786 7621            | 6424    | 5284 7522           | 0.200 |
| Myristic acid                     | 3010  | 2509 3729            | 2848    | 2429 3582           | 0.532 |
| Nonanoic acid                     | 6932  | 4885 9101            | 7186    | 4448 9252           | 0.911 |
| Oleic acid                        | 17934 | 13900 25612           | 16020   | 10955 20360          | 0.017 |
| O-Phosphoethanolamine             | 1992  | 1562 2480            | 2190    | 1846 2646           | 0.046 |
| Oxalacetic acid + Pyruvate        | 3616  | 2321 5150            | 4453    | 2552 6365           | 0.104 |
| Palmitic acid                     | 90410 | 79816 108730          | 86366   | 77289 100930         | 0.172 |
| Palmitoleic acid                  | 1759  | 1329 2432            | 1395    | 1016 1792           | 0.003 |
| Phenylalanine                     | 15242 | 12430 19375           | 16423   | 12646 19168          | 0.705 |
| Phosphate                         | 225161 | 206741 247737         | 217909  | 186908 235184        | 0.026 |
| Proline                           | 72054 | 53178 98092           | 84497   | 62222 113453         | 0.147 |
(Cont. Supplementary Table 2)

| metabolite                  | Non-CVD subjects (n = 176) |           |           | CAD subjects (n = 40) |           |           | p-value |
|-----------------------------|----------------------------|-----------|-----------|----------------------|-----------|-----------|---------|
|                             | median                     | Interquartile range |           | median              | Interquartile range |           |         |
|                             | 25th | 75th | 25th | 75th | 25th | 75th |         |
| Psicose + tagatose          | 1775 | 1398 | 2341 | 1527 | 1269 | 1846 | 0.014 |
| Pyroglutamic acid           | 32232 | 27973 | 35242 | 32286 | 28370 | 36187 | 0.775 |
| Quinic acid                 | 235 | 91 | 731 | 485 | 108 | 1570 | 0.024 |
| Serine 2TMS                 | 4651 | 3326 | 6239 | 4859 | 3068 | 6069 | 0.693 |
| Serine 3TMS                 | 26657 | 20585 | 34294 | 22365 | 19198 | 29570 | 0.027 |
| Stearic acid                | 46435 | 39083 | 53480 | 46146 | 39630 | 51380 | 0.545 |
| Sucrose                     | 596 | 258 | 941 | 608 | 409 | 1088 | 0.424 |
| Threonic acid               | 2349 | 1655 | 3175 | 2023 | 1575 | 2940 | 0.170 |
| Threonine 2TMS              | 3175 | 2339 | 4491 | 3556 | 2740 | 4460 | 0.316 |
| Threonine 3TMS              | 16281 | 12816 | 22019 | 16189 | 13770 | 21672 | 0.944 |
| Tyrosine                    | 35074 | 28390 | 47541 | 36322 | 29663 | 45454 | 0.998 |
|         | 39753 | 32535 | 48021 | 38201 | 31746 | 45154 | 0.433 |
| Urea                        | 1541963 | 1285519 | 1925256 | 1751858 | 1444443 | 2230490 | 0.035 |
| Uric acid                   | 91107 | 70301 | 111952 | 94185 | 77554 | 109324 | 0.338 |
| Valine                      | 145590 | 119908 | 180544 | 130039 | 108568 | 170454 | 0.109 |
| Unknown 0                   | 824 | 600 | 1180 | 724 | 513 | 1278 | 0.668 |
| Unknown 1                   | 7678 | 6046 | 8847 | 7615 | 6589 | 8768 | 0.973 |
| Unknown 2                   | 3713 | 3351 | 4110 | 3713 | 3269 | 4126 | 0.996 |
| Unknown 4                   | 1444 | 1055 | 1922 | 1667 | 1200 | 2426 | 0.052 |
| Unknown 5                   | 20436 | 19651 | 21336 | 20474 | 19421 | 21554 | 0.911 |
| Unknown 6                   | 30033 | 25302 | 35751 | 28637 | 24405 | 35684 | 0.573 |
| Unknown 8                   | 7208 | 6693 | 8015 | 7315 | 6840 | 7912 | 0.873 |
| Unknown 9                   | 7380 | 6026 | 8570 | 7334 | 6463 | 8871 | 0.560 |
| Unknown 11                  | 3295 | 2937 | 3611 | 3489 | 2863 | 3756 | 0.234 |
| Unknown 13                  | 92429 | 88679 | 95685 | 92358 | 89872 | 95999 | 0.592 |
| Unknown 14                  | 667 | 330 | 1203 | 952 | 458 | 2197 | 0.060 |
| Unknown 15                  | 5885 | 5021 | 7242 | 5546 | 4869 | 7181 | 0.915 |
| Unknown 17                  | 698 | 48 | 1091 | 852 | 439 | 1030 | 0.838 |
| Unknown 20                  | 66400 | 56814 | 79105 | 70103 | 60579 | 89787 | 0.212 |
| Unknown 21                  | 31409 | 30214 | 32933 | 31494 | 30007 | 33129 | 0.882 |
| Unknown 25                  | 16044 | 15037 | 17132 | 16498 | 15314 | 16960 | 0.349 |
| Unknown 26                  | 927221 | 867528 | 1033880 | 937809 | 862018 | 1037918 | 0.877 |
| Unknown 27                  | 5404 | 5021 | 5958 | 5165 | 4730 | 5795 | 0.089 |
| Unknown 28                  | 4866 | 4414 | 5475 | 5117 | 4711 | 5528 | 0.084 |
| Unknown 29                  | 2425 | 722 | 5635 | 5425 | 718 | 9517 | 0.030 |
| Unknown 31                  | 45355 | 28440 | 89191 | 44943 | 24936 | 76634 | 0.297 |
| Unknown 32                  | 1244 | 948 | 1639 | 1354 | 1105 | 1909 | 0.098 |
| Unknown 33                  | 2676 | 2282 | 3060 | 2740 | 2275 | 3221 | 0.443 |
| Unknown 34                  | 2794 | 2286 | 3959 | 2391 | 1788 | 3697 | 0.079 |
| Unknown 35                  | 4499 | 3983 | 5484 | 4429 | 3847 | 5410 | 0.695 |
| Unknown 38                  | 4016 | 3410 | 4820 | 5185 | 4133 | 6659 | 0.000 |
| Unknown 39                  | 2430 | 2096 | 2830 | 2390 | 1982 | 2767 | 0.464 |
| Unknown 40                  | 2101 | 1789 | 2513 | 2084 | 1708 | 2489 | 0.650 |
| Unknown 41                  | 5007 | 3987 | 6120 | 4780 | 3618 | 6536 | 0.714 |
| Unknown 44                  | 3401 | 2817 | 4149 | 3519 | 2719 | 4349 | 0.792 |
| Unknown 45                  | 24513 | 23202 | 25754 | 23769 | 21629 | 25022 | 0.041 |
| Unknown 46                  | 3832 | 3253 | 4499 | 3545 | 3112 | 4448 | 0.314 |
| Unknown 47                  | 8369 | 6334 | 12052 | 8528 | 6693 | 11168 | 0.931 |
| Unknown 53                  | 2062 | 1803 | 2287 | 2015 | 1774 | 2360 | 0.962 |
| Unknown 58                  | 2379 | 2091 | 2701 | 2456 | 2197 | 2891 | 0.145 |
| Unknown 67                  | 2541 | 2037 | 3144 | 2503 | 1958 | 2918 | 0.560 |
(Cont. Supplementary Table 2)

| metabolite | Non-CVD subjects (n = 176) | CAD subjects (n = 40) | p-value |
|------------|---------------------------|-----------------------|---------|
|            | median                    | Interquartile range   | median  | Interquartile range |         |
|            | 25th 75th                 | 25th 75th             |         |                     |         |
| Unknown 68 | 2050 1615 2620            | 1951 1559 2494        | 0.525   |
| Unknown 70 | 590 373 804               | 690 508 922           | 0.032   |
| Unknown 71 | 6583 5759 7305            | 6365 5844 6796        | 0.165   |
| Unknown 73 | 485 301 761               | 640 459 858           | 0.005   |
| Unknown 74 | 1894 1452 2388            | 2048 1625 2460        | 0.187   |
| Unknown 76 | 6064 5634 6599            | 6254 5406 6772        | 0.697   |
| Unknown 78 | 14276 13389 15098         | 14133 13232 14757     | 0.349   |
| Unknown 83 | 1289 831 2063             | 1200 832 1494         | 0.277   |
| Unknown 85 | 1950 1402 2525            | 2246 1836 3080        | 0.008   |
| Unknown 86 | 3165 2368 4062            | 3667 2574 4239        | 0.157   |
| Unknown 87 | 1933 1525 2553            | 2281 1832 2833        | 0.013   |
| Unknown 88 | 2021 1304 2735            | 2243 1729 3775        | 0.024   |
| Unknown 89 | 2891 2157 3626            | 3041 2209 3558        | 0.884   |
| Unknown 93 | 3167 2555 3902            | 3571 3053 4332        | 0.023   |
| Unknown 94 | 7541 6075 9474            | 7062 6305 10443       | 0.944   |
| Unknown 95 | 1936 1304 2555            | 1842 1196 2336        | 0.569   |
| Unknown 98 | 4408 2106 5816            | 4498 1739 5784        | 0.803   |
| Unknown 101| 438 280 611               | 424 261 709           | 0.814   |
| Unknown 105| 3415 2622 4207            | 4189 3443 5073        | 0.000   |
| Unknown 106| 2853 2142 3756            | 3416 2890 4246        | 0.006   |
| Unknown 108| 1731 1316 2391            | 1776 1353 2518        | 0.953   |
| Unknown 111| 3218 2578 3933            | 3392 2270 4115        | 0.996   |
| Unknown 115| 818 575 1131              | 852 593 1076          | 0.840   |
| Unknown 120| 13541 10769 16788         | 12631 10244 16018     | 0.408   |
| Unknown 131| 3514 2022 8144            | 3164 2084 10599       | 0.842   |
| Unknown 145| 17155 13655 22086         | 16896 13778 20589     | 0.648   |
| Unknown 146| 2631 1343 3887            | 2736 1815 3394        | 0.528   |
| Unknown 147| 13319 7759 21580          | 10015 7154 21645      | 0.431   |
| Unknown 148| 1182 938 1519             | 1215 995 1496         | 0.760   |
| Unknown 153| 48689 40814 63431         | 46375 3792 56612      | 0.287   |
| Unknown 154| 260 101 459              | 333 129 655           | 0.281   |
| Unknown 155| 3397 2689 4408            | 3169 2386 3909        | 0.225   |
| Unknown 159| 1716 1265 2237            | 1982 1169 2584        | 0.287   |
| Unknown 161| 3770 3169 4828            | 3435 2710 3976        | 0.010   |
| Unknown 162| 1958 1476 2524            | 1982 1769 2421        | 0.383   |
| Unknown 170| 1245 990 1642            | 1501 1217 2430        | 0.004   |
| Unknown 173| 668688 540992 777006      | 633308 483239 785793  | 0.396   |
| Unknown 175| 2714 2301 3125            | 2635 2232 3086        | 0.632   |
| Unknown 176| 2203 1815 2546            | 2339 2068 2693        | 0.035   |
| Unknown 177| 1176 869 1604            | 1028 800 1632         | 0.367   |
| Unknown 178| 4183 3692 4703            | 4064 3558 4597        | 0.652   |
| Unknown 182| 2771 2223 3747            | 2673 2093 3770        | 0.716   |
| Unknown 185| 4027 3082 5147            | 3933 3174 4993        | 0.989   |
| Unknown 186| 4305 3615 5551            | 4598 3702 4987        | 0.730   |
| Unknown 187| 3070 2466 3908            | 2863 2499 3707        | 0.840   |
| Unknown 190| 3632 2970 4560            | 3422 2946 4366        | 0.583   |
| Unknown 193| 2853 2307 3560            | 2789 2201 3498        | 0.880   |
| Unknown 197| 2185 1734 2671            | 2329 2041 2614        | 0.208   |
| Unknown 198| 1888 1538 2499            | 1737 1424 2161        | 0.140   |
Supplementary Table 3. List of unknown metabolites significantly associated with either max-IMT or FMD in non-CVD subjects

| Metabolite   | FMD (n = 154) | max-IMT (n = 165) |
|--------------|--------------|------------------|
|              | ρ      | p-value | ρ      | p-value |
| Unknown 2    | -0.169 | 0.036   | -0.019 | 0.813   |
| Unknown 9    | -0.240 | 0.003   | -0.294 | 1.3 × 10⁻⁴ |
| Unknown 17   | -0.169 | 0.036   | 0.251  | 0.001   |
| Unknown 29   | -0.144 | 0.075   | 0.245  | 0.002   |
| Unknown 38   | -0.164 | 0.042   | 0.246  | 0.001   |
| Unknown 41   | -0.063 | 0.441   | 0.201  | 0.010   |
| Unknown 76   | 0.072  | 0.372   | 0.156  | 0.002   |
| Unknown 83   | -0.163 | 0.044   | 0.066  | 0.400   |
| Unknown 87   | 0.004  | 0.963   | -0.155 | 0.046   |
| Unknown 95   | 0.032  | 0.696   | 0.224  | 0.004   |
| Unknown 105  | 0.052  | 0.525   | 0.201  | 0.010   |
| Unknown 106  | -0.003 | 0.974   | 0.229  | 0.003   |
| Unknown 120  | -0.165 | 0.041   | 0.220  | 0.004   |
| Unknown 135  | -0.178 | 0.027   | 0.091  | 0.244   |
| Unknown 155  | -0.077 | 0.344   | 0.155  | 0.046   |
| Unknown 162  | -0.217 | 0.007   | 0.006  | 0.942   |
| Unknown 170  | -0.146 | 0.072   | 0.217  | 0.005   |
| Unknown 193  | 0.014  | 0.863   | 0.187  | 0.016   |
| Unknown 197  | -0.010 | 0.906   | -0.154 | 0.048   |
| Unknown 198  | 0.178  | 0.027   | 0.167  | 0.032   |

Spearman’s rank correlation coefficient was evaluated to detect the metabolites associated with the FMD or max-IMT. Bold font indicates statistically significant (p < 0.05) difference.

Supplementary Table 4. Clinical characteristics of subjects with and without CAD matched with propensity score

|                        | Non-CVD subjects | CAD subjects |
|------------------------|------------------|--------------|
| Number of subjects     | 77               | 31           |
| Age (years)            | 64.8 ± 7.7       | 66.5 ± 6.0   |
| Male gender (n, %)     | 45 (58.4)        | 21 (67.7)    |
| BMI (kg/m²)            | 27.2 ± 4.6       | 26.7 ± 4.8   |
| eGFR (ml/min/1.73 m²)  | 67.7 ± 23.0      | 61.7 ± 19.2  |
| Diabetes duration (years) | 15.0 ± 9.4    | 17.2 ± 11.5  |
| HbA1c (%)              | 8.7 ± 1.4        | 8.4 ± 1.6    |
| Smoking history (n, %) | 42 (54.5)        | 17 (54.8)    |
| Hypertension (n, %)    | 68 (88.3)        | 30 (96.8)    |
| Systolic BP (mmHg)     | 127 ± 20         | 121 ± 16     |
| Dyslipidemia (n, %)    | 58 (75.3)        | 28 (90.3)    |
| Total cholesterol (mg/dl) | 191 ± 36      | 172 ± 41     |
| HDL cholesterol (mg/dl)| 49.4 ± 12.4     | 50.3 ± 19.2  |
| LDL cholesterol (mg/dl)| 107 ± 28        | 90 ± 24      |
| Triglyceride (mg/dl)   | 171 ± 117        | 182 ± 126    |
| Medication use         |                  |              |
| Diabetes (n, %)        | 70 (90.9)        | 27 (90.3)    |
| Hypertension (n, %)    | 68 (88.3)        | 30 (96.8)    |
| Dyslipidemia (n, %)    | 49 (63.6)        | 27 (87.1)    |

Data are presented as mean ± standard deviation or number with percentage in parentheses. Matching variables were age, gender, diabetes duration, eGFR, HbA1c, smoking history, hypertension, hyperlipidemia, and medication use for diabetes, hypertension, and hyperlipidemia (1:n matching).