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Original Article

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Identification of Plasma Inositol and Indoxyl Sulfate as Novel Biomarker Candidates for Atherosclerosis in Patients with Type 2 Diabetes. -Findings from Metabolome Analysis Using GC/MS-

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Aim: An identification of the high-risk group of atherosclerotic cardiovascular disease (CVD) is important in the management of patients with diabetes. Metabolomics is a potential tool for the discovery of new biomarkers. With this background, we aimed to identify metabolites associated with atherosclerosis in patients with type 2 diabetes mellitus (T2DM).

Methods: A total of 176 patients with T2DM who have never had a CVD event and 40 who were survivors of coronary artery disease (CAD) events were enrolled. Non-targeted metabolome analysis of fasting plasma samples was performed using gas chromatography coupled with mass spectrometry (GC/MS) highly optimized for multiple measurement of blood samples. First, metabolites were screened by analyzing the association with the established markers of subclinical atherosclerosis (i.e., carotid maximal intima-media thickness (max-IMT) and flow-mediated vasodilation (FMD)) in the non-CVD subjects. Then, the associations between the metabolites detected and the history of CAD were investigated.

Result: A total of 65 annotated metabolites were detected. Non-parametric univariate analysis identified inositol and indoxyl sulfate as significantly ($p<0.05$) associated with both max-IMT and FMD. These metabolites were also significantly associated with CAD. Moreover, inositol remained to be associated with CAD even after adjustments for traditional coronary risk factors.

Conclusions: We identified novel biomarker candidates for atherosclerosis in Japanese patients with T2DM using GC/MS-based non-targeted metabolomics.

Key words: Metabolomics, Coronary artery disease, Intima-media thickness (IMT), Flow-mediated dilation (FMD), Diabetes

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

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in the management of patients with diabetes. However, there are significant limitations in cardiovascular risk assessment based on conventional risk factors[2-4], 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 metabolomic 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[5], 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[6]. At the same time, fasting plasma used for metabolomics analysis was collected and cooled immediately in a freezer at 4℃. It was then centrifuged (3,000×g, 10 min) and stored at −80℃ 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[7]. The estimated glomerular filtration rate (eGFR mL/min/1.73 m^2) was calculated using an equation proposed by the Japanese Society of Nephrology[8].

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[9]. 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[10-12] 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: FMD (%) = (maximum diameter - diameter at rest) × 100/ diameter at rest.

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 H_2O containing 0.2 mg/mL of ribitol, which was used as an internal standard (1,200 rpm, 10 min, 4℃). Then, 800 µL of deaerated MeCN was added (1200 rpm, 10 min, 4℃) and centrifuged (16,000 × g, 3 min, 4℃). 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℃.

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℃). A second derivatizing agent, *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide, was then added and the mixture was incubated (1,200 rpm, 30 min, 37℃). After centrifugation (16,000 × 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℃. The helium gas flow rate through the column was 1.12 mL/min. The column temperature was set to 80℃ for 2 min and then raised to 330℃ for 12 min. The temperature of the transfer interface and ion source was set to 310℃ and 280℃, 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 decafluorotriphenylphosphine (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[13]. 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 (http://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[14], 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/m^2)	27.7 ± 5.9	26.4 ± 4.6, *n* = 37	0.212
eGFR (ml/min/1.73 m^2)	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.
BMI, body mass index; FPG, fasting plasma glucose; BP, blood pressure; GFR, glomerular filtration rate

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.

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).

Results

Patient Characteristics

Patient characteristics of the non-CVD subjects

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

Metabolite	FMD ($n=154$)		max-IMT ($n=165$)	
	ρ	p-value	ρ	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	$\mathbf{9.5\times10^{-5}}$
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)	$\mathbf{2.8\times10^{-5}}$
Inositol	10210 (8360-12620)	12830 (10590-16250)	$\mathbf{2.6\times10^{-4}}$

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

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 subjcts 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.

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 [95% confidence interval {CI}] 1.43 [1.05–1.95], $p=0.022$) 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[15], 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 and previous studies[16, 17] showed that MeCN was highly efficient in protein removal compared with other organic solvents. Moreover, the extraction at 4℃ 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℃ 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[18]. Carotid IMT has indeed been shown to be a predictor of CVD[19, 20]. In contrast, FMD is a marker of early atherosclerosis causing endothelial dysfunction and is also known as a predic-

tor of CVD[21, 22]. 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[23]. 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[24]. 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[25, 26]. 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[27], 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[28-30]. 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[30, 31], 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[32]. 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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None.

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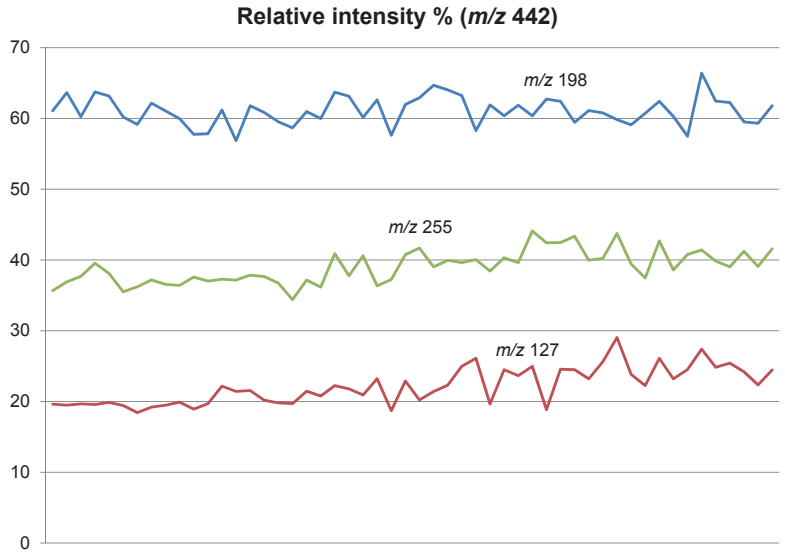

Supplementary Fig. 1. Relative intensity of each fragment of DFTPP

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 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℃). Next, 400 μL of water was added and mixed. After further centrifugation (16,000 g, 3 min, 4℃), 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℃). Subsequently, 800 μL of the organic solvent was added and mixed (1,200 rpm, 30 min, 4℃). After centrifugation (16,000 g, 3 min, 4℃), 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℃). 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:H_2O:$CHCl_3$ (5:1:2)	15.5 ± 2.2
H_2O:MeCN (1:4)	0.5 ± 0.3
H_2O:MeOH (1:4)	3.3 ± 0.9

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		median	Interquartile range		
		25th	75th		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-Hydroxypyridine	3953	3587	4369	3976	3448	4531	0.955
3-Amino isobutyric acid	1664	962	2343	1882	977	2559	0.498
Alanine 2TMS	118231	96391	164060	121565	91122	183365	0.610
Alanine 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	69094	52267	100717	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
Isocitric 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
Nonanoric 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	
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
Tryptophan	35074	28390	47541	36322	29663	45454	0.998
Tyrosine	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	481	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 ($n=176$) median	Non-CVD subjects ($n=176$) Interquartile range 25th	Non-CVD subjects ($n=176$) Interquartile range 75th	CAD subjects ($n=40$) median	CAD subjects ($n=40$) Interquartile range 25th	CAD subjects ($n=40$) Interquartile range 75th	*p*-value
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	37992	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	0.193	**0.017**	-0.019	0.813
Unknown 9	-0.169	**0.036**	0.102	0.193
Unknown 17	-0.240	**0.003**	0.294	**1.3×10^{-4}**
Unknown 29	-0.169	**0.036**	0.251	**0.001**
Unknown 38	-0.144	0.075	0.245	**0.002**
Unknown 41	-0.164	**0.042**	0.246	**0.001**
Unknown 76	-0.063	0.441	0.201	**0.010**
Unknown 83	-0.072	0.372	0.156	**0.002**
Unknown 87	-0.163	**0.044**	0.066	0.400
Unknown 95	0.004	0.963	-0.155	**0.046**
Unknown 105	0.032	0.696	0.224	**0.004**
Unknown 106	0.052	0.525	0.201	**0.010**
Unknown 120	-0.003	0.974	0.229	**0.003**
Unknown 146	-0.165	**0.041**	0.220	**0.004**
Unknown 153	-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/m^2)	27.2 ± 4.6	26.7 ± 4.8
eGFR (ml/min/1.73 m^2)	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).