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Identification of Metabolites Associated with Onset of CAD in Diabetic Patients Using CE-MS Analysis: A Pilot Study

Kazuo Omori[1], Naoto Katakami[1, 2], Yuichi Yamamoto[1], Hiroyo Ninomiya[1], Mitsuyoshi Takahara[1, 3], Taka-aki Matsuoka[1], Takeshi Bamba[4], Eiichiro Fukusaki[5] and Iichiro Shimomura[1]

[1]Department of Metabolic Medicine, Osaka University Graduate School of Medicine, Osaka, Japan
[2]Department of Metabolism and Atherosclerosis, Osaka University Graduate School of Medicine, Osaka, Japan
[3]Department of Diabetes Care Medicine, Graduate School of Medicine, Osaka University, Osaka, Japan
[4]Division of Metabolomics, Medical Institute of Bioregulation, Kyushu University, Fukuoka Japan
[5]Laboratory of Bioresource Engineering, Department of Biotechnology, Graduate School of Engineering, Osaka University, Osaka, Japan

Aim: Coronary artery disease (CAD) is the result of a complex metabolic disorder caused by various environmental and genetic factors. Metabolomics is a potential tool for identifying biomarkers for better risk classification and for understanding the pathophysiological mechanisms of CAD. With this background, we performed a pilot study to identify metabolites associated with the future onset of CAD in patients with type 2 diabetes.

Methods: Sixteen subjects who suffered from CAD event during the observation period and 39 non-CAD subjects who were matched to the CAD subjects for Framingham Coronary Heart Disease Risk Score, diabetes duration, and HbA1c were selected. Capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) was used to perform non-targeted metabolome analysis of serum samples collected in 2005.

Results: A total of 104 metabolites were identified. Unsupervised principal component analysis (PCA) did not to reveal two distinct clusters of individuals. However, a significant association with CAD was found for 7 metabolites (pelargonic acid, glucosamine:galactosamine, thymine, 3-hydroxybutyric acid, creatine, 2-aminoisobutyric acid, hypoxanthine) and the levels of all these metabolites were significantly lower in the CAD group compared with the non-CAD group.

Conclusions: We identified 7 metabolites related to long-term future onset of CAD in Japanese patients with diabetes. Further studies with large sample size would be necessary to confirm our findings, and future studies using *in vivo* or *in vitro* models would be necessary to elucidate whether direct relationships exist between the detected metabolites and CAD pathophysiology.

Key words: Metabolomics, Coronary artery disease, Diabetes

Introduction

Coronary artery disease (CAD) is one of the leading causes of morbidity and mortality worldwide in patients with type 2 diabetes (T2DM)[1, 2]. In the management of overall health in diabetic patients, early and accurate identification of the groups at high risk of CAD is important. However, cardiovascular risk assessment based on conventional risk factors such as age, sex, smoking status, blood pressure, and cholesterol showed only moderate performance[3-5]. Thus, the exploration of new molecules or pathways related to considerable residual risk of CAD is needed. Since CAD is the result of complex metabolic disorders caused by various environmental and genetic factors, an approach to evaluate comprehensive metabolic change could be helpful to increase the mechanistic understanding of atherosclerosis and to identify indicators for high-risk patients.

Metabolomics, the study of small-molecule metab-

Address for correspondence: Naoto Katakami, Department of Metabolic Medicine, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan E-mail: katakami@endmet.med.osaka-u.ac.jp

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olites that are by-products of a variety of biochemical and cellular processes, is one of the most hopeful omics techniques, and depicts the real-time metabolic status of an organism[6, 7]. Therefore, metabolomics is a potential tool for identifying biomarkers for better risk classification and for understanding the pathophysiological mechanism of cardiovascular diseases. Non-targeted metabolomic profiling refers to the identification of metabolites using a hypothesis-free approach and, hence, would be useful for discovering novel metabolites.

Capillary electrophoresis-mass spectrometry (CE-MS) is a commonly used tool in metabolome analysis. The major advantage of CE-MS is high separation efficiency and high ability to identify and quantify global polar ionogenic metabolites, such as carbohydrates and amino acids[8, 9]. Therefore, CE-MS systems have been used in many clinical studies to evaluate metabolic fingerprinting of various biological samples for biomarker discovery and understanding the pathophysiology of diseases[10-13].

With this background, we performed a pilot study to identify metabolites associated with future onset of CAD in patients with T2DM using CE-MS analysis.

Methods

Subjects

The current study was based on data retrospectively obtained from the Order-Made multiple Risk Factor Investigation Trial (OMRFIT), which was a prospective multicenter cohort study to investigate the genetic factors for diabetic complications in 4777 Japanese diabetic patients. In the original study, 473 subjects were registered at Osaka University Hospital during January-December 2005 and followed up until 2013 (median follow up period; 8.7 years). In the present study, the subjects were recruited from these 473 subjects and written informed consent was obtained from 176 subjects. Among them, 16 subjects who developed CAD events during the observation period were selected as the CAD-positive group. For this pilot study, a non-CAD control group consisting of 39 subjects was matched to the CAD-positive group for Framingham Coronary Heart Disease Risk Score, diabetes duration, and HbA1c, which are assumed to be clinical factors with a large influence on CAD. The procedure was as follows. After excluding 10 out of 160 non-CAD patients because of missing data, the process of selecting 39 subjects was continued until three factors with a standardized difference of 0.58 or less were found. This cut-off value of standardized difference was calculated based on the formula previously reported ($1.96\sqrt{(n_C + n_N)/n_C n_N}$, n_C, n_N: number of patients, respectively, in the CAD and non-CAD groups)[14, 15]. The standardized difference in FRS, diabetes duration, and HbA1c between CAD-positive group and selected 39 non-CAD subjects was 0.25, 0.10, and 0.06, respectively.

In the present study, a CAD event was defined as cardiovascular death, acute myocardial infarction, angina pectoris, or coronary revascularization treatment that included coronary intervention and coronary bypass graft. The diagnosis of the occurrence of coronary artery disease was performed by cardiologists based on the clinical symptoms, characteristic ECG changes, cardiac enzyme levels, and findings on coronary angiography and/or echocardiography, according to established guidelines[16, 17].

Ethics, Consent, and Permissions

The Research Ethics Committee of Osaka University Graduate School of Medicine approved the study protocol 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.

Clinical and Biochemical Analysis

The clinical and biochemical data at baseline were collected in 2005, the start year of OMRFIT, a longitudinal observation study. Fasting blood samples were collected and HbA1c and serum total and HDL-cholesterol levels were measured using standard laboratory protocols. The following criteria was used to diagnose diabetes: early-morning fasting plasma glucose (PG) ≥ 126 mg/dl, two-hour PG after 75 g glucose load ≥ 200 mg/dl, or causal plasma glucose load ≥ 200 mg/dl. The determination of hypertension (defined as systolic blood pressure (SBP) ≥ 130 mmHg or diastolic blood pressure (DBP) ≥ 80 mmHg or anti-hypertensive medication use) and dyslipidemia (defined as serum LDL cholesterol ≥ 3.1 mmol/L (120 mg/dl) or serum TG ≥ 1.7 mmol/L (150 mg/dl) or HDL-cholesterol < 1.0 mmol/L (40 mg/dl) or lipid-lowering medication use) was based on the criteria of the Japan Diabetes Society. The risk of CAD was estimated by FRS, which uses information on age, gender, total and HDL-cholesterol level, blood pressure, smoking status, and the existence of diabetes mellitus[18].

Capillary Electrophoresis Time-of-Flight Mass Spectrometry (CE-TOFMS) Analysis

For the metabolome analysis, the frozen stored serum that was collected in 2005, the start year of the longitudinal observation before the development of CAD event, was used. The CE-TOFMS measurement was performed in April 2014 at Human Metabolome

Technologies, Inc., Tsuruoka, Japan, using the baseline fasting serum samples that had been stored at −80℃ since collection. The CE-TOFMS analysis was operated using the cation-positive mode and anion-negative mode. The serum samples were thawed on ice before preparation. For the extraction of serum metabolites, 50 μl serum samples were added to 450 μl of methanol containing internal standards (Solution ID: H3304-1002, Human Metabolome Technologies, Inc., Tsuruoka, Japan). Then, the samples were mixed with 200 μl Milli-Q water and 500 μl chloroform and were centrifuged at 2300 ×g at 4℃ for 5 min. Subsequently, 350 μl of the upper aqueous solution was centrifugally filtered through 5-kDa cut-off filter (Millipore) at 9100 ×g at 4℃ for 120 min. The filtrate (400 μl) was centrifugally concentrated and was re-suspended 25 μl Milli-Q water for the CE-TOFMS analysis.

Data Processing

Peaks were extracted using MasterHands (Keio University, Tsuruoka, Japan) automatic integration software to obtain peak information, including *m/z*, migration time for CE-TOFMS measurement (MT), and peak area. Signal peaks corresponding to isotopomers, adduct ions, and other product ions of known metabolites were excluded, and the remaining peaks were annotated with putative metabolites from the HMT metabolite database based on their MTs and *m/z* values determined by TOFMS. The tolerance range for the peak annotation was configured at ±0.5 min for MT and ±10 ppm for *m/z*. In addition, peak areas were normalized against those of the internal standard for cations (MetSul [methionine sulfone]) and that for anions (CSA [D-camphor-10-sulfonic acid]) and, then, the resultant relative area values were further normalized to the sample amount.

Statistical Analysis

The data are reported as mean±standard deviation (SD) for continuous variables and percentage for dichotomous variables. Unpaired *t*-test was used to compare the means. Pearson's chi-squared test was used to compare the categorical variables. Principal component analysis (PCA) and Welch's *t*-test were used to analyze the relationships between CAD and the levels of serum metabolites. $p<0.05$ was considered statistically significant. The prediction ability of each detected metabolite to discriminate between subjects with and without CAD was examined by receiver-operating-characteristic (ROC) curve analyses. SPSS version 22 (SPSS Inc., Chicago, IL, USA) was used to perform these statistical analyses.

Results

Clinical Characteristics of the Study Population

CAD events occurred in 16 subjects out of 176 (9.1%) during the observation period, and from the 160 subjects without CAD, 39 control subjects who were matched to the CAD group for FRS, diabetes duration, and HbA1c were selected (**Supplementary Fig. 1**). **Table 1** lists the baseline clinical characteristics of the study subjects with and without the new onset of CAD during the observation period. Among the subjects who experienced CAD during follow up (males, 88%; age, 66.3±6.1 years; diabetes duration, 17.2±10.1 years; HbA1c, 7.1±0.7%), 10 (63%) subjects had hypertension, 10 (63%) had dyslipidemia, and 8 (53%) had a smoking habit. There were no significant differences in the majority of the clinical variables between the two groups. Half of the subjects (8 of 16) in the CAD group had a history of coronary intervention or coronary artery bypass graft (CABG), whereas no subject of the non-CAD group had history of coronary artery treatment.

Metabolomic Profiling

A total of 104 metabolites were identified from the serum samples after excluding the components of therapeutic medicines and their metabolites, substances suspected as contaminants (e.g., eluted substances from sample storage tubes), and metabolites with unknown identities. Before further analysis, 8 metabolites which were not detected in ≥25% of the samples in each group were excluded. Consequently, 96 metabolites were analyzed for association with CAD (**Supplementary Table 1**).

Metabolites Associate with CAD

Unsupervised PCA was performed to check whether group differentiation was achievable according to the metabolite profiles (**Supplementary Fig. 2**). However, the PCA did not reveal two distinct clusters of individuals, which would be expected if the sampled individuals belonged to two different groups. Then, Welch's *t*-test was used to investigate the association of each metabolite with CAD. A significant association with CAD was found in 7 metabolites (pelargonic acid, glucosamine:galactosamine, thymine, 3-hydroxybutyric acid, creatine, 2-aminoisobutyric acid, hypoxanthine) out of 96, and the levels of all of these metabolites were significantly lower in the CAD group compared with the non-CAD group (**Fig. 1**).

Next, ROC curve analyses were performed to quantify the ability of these 7 metabolites to predict CAD onset (**Table 2**). These analyses revealed that pelargonic acid and 3-hydroxybutyric acid showed high

Table 1. Baseline clinical characteristics of study subjects with and without new onset of CAD during the observation period

Characteristics	Non-CAD group ($n=39$)	CAD group ($n=16$)	p value
Age (years)	63.7 ± 8.2	66.3 ± 6.1	0.271
Male gender (n (%))	29 (74)	14 (88)	0.284
Diabetes duration (years)	16.2 ± 10.0	17.2 ± 10.1	0.990
BMI (kg/m^2)	24.4 ± 3.1	25.1 ± 3.3	0.469
HbA1c (%)	7.1 ± 1.0	7.1 ± 0.7	0.841
Smoking (n (%))	20 (51)	8 (53)	0.893
Hypertension (n (%))	33 (85)	10 (63)	0.071
Systolic BP (mmHg)	138.6 ± 14.2	130.4 ± 21.1	0.101
Diastolic BP (mmHg)	78.2 ± 12.6	71.8 ± 12.2	0.086
Dyslipidemia (n (%))	28 (72)	10 (63)	0.498
HDL cholesterol (mg/dl)	54.9 ± 24.4	47.7 ± 13.6	0.316
LDL cholesterol (mg/dl)	116.9 ± 31.7	109.1 ± 24.8	0.394
Framingham risk score (%)	24.3 ± 12.4	27.4 ± 11.2	0.392
Medication use			
ACE inhibitor/ARB (n (%))	11 (28)	7 (44)	0.264
Statin (n (%))	28 (72)	10 (63)	0.498
Insulin (n (%))	13 (33)	9 (56)	0.115
Antiplatelet agent (n (%))	23 (59)	11 (69)	0.498

Data are presented as mean ± standard deviation or number with percentage in parentheses. BMI, body mass index; BP, blood pressure; ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker

C-statistic values (0.801, 95% confidential interval (CI); 0.697−0.915, $p<0.001$ and 0.716, 95% CI; 0.567−0.866, $p=0.012$, respectively), indicating that these metabolites were useful in the risk estimation for CAD.

Discussion

To reduce mortality in the management of diabetes, early prediction of coronary artery disease, and subsequent rapid intervention, are important. Although cardiovascular risk assessment and prediction are performed based on conventional risk factors, validation studies have shown that this approach had only moderate performance[3-5]. Therefore, detection of unknown pathways related to the progression of atherosclerosis and identification of new prognostic markers for CAD could improve the quality and efficiency of management of overall health in diabetic patients.

In the present pilot study, 104 metabolites were quantified in 55 diabetic subjects with or without CAD by metabolome analysis using CE-TOFMS. Among these metabolites, 7 substrates (pelargonic acid, glucosamine:galactosamine, thymine, 3-hydroxybutyric acid, creatine, 2-aminoisobutyric acid, hypoxanthine) were significantly associated with CAD, and all of these 7 metabolites were lower in the CAD group compared with control subjects. These findings suggest that these 7 metabolites could be novel biomarkers for future onset of CAD. In particular, pelargonic acid and 3-hydroxybutric acid may be promising surrogate markers, because they had discriminatory ability to predict CAD onset in ROC analysis. Furthermore, some of them may play crucial roles in the pathogenesis of CAD.

A few studies have been reported in relevance to the substrates detected in our study and to atherosclerotic disease. Glucosamine is a naturally occurring amino monosaccharide and well known as a precursor for glycosaminoglycans and mucopolysaccharides, major components of joint cartilage, connective tissue, skin, and tendons. There are few data regarding the direct effect of glucosamine on atherosclerotic diseases, and those studies showed controversial results[19-24]. Some *in vivo* and *in vitro* studies reported protective roles of glucosamine against atherosclerosis with anti-inflammatory effect or inhibition of smooth muscle cell growth[19-21], while other studies showed that glucosamine accelerates atherosclerotic change[22] or endoplasmic reticulum stress[23, 24]. Our data may support the anti-atherosclerotic effect of glucosamine, because its level was significantly lower in patients who developed CAD during the observation period.

Both 3-hydroxybutyric acid and creatine play important roles in energy metabolism. 3-hydroxybutyric acid is a ketone body that is raised in ketosis and can

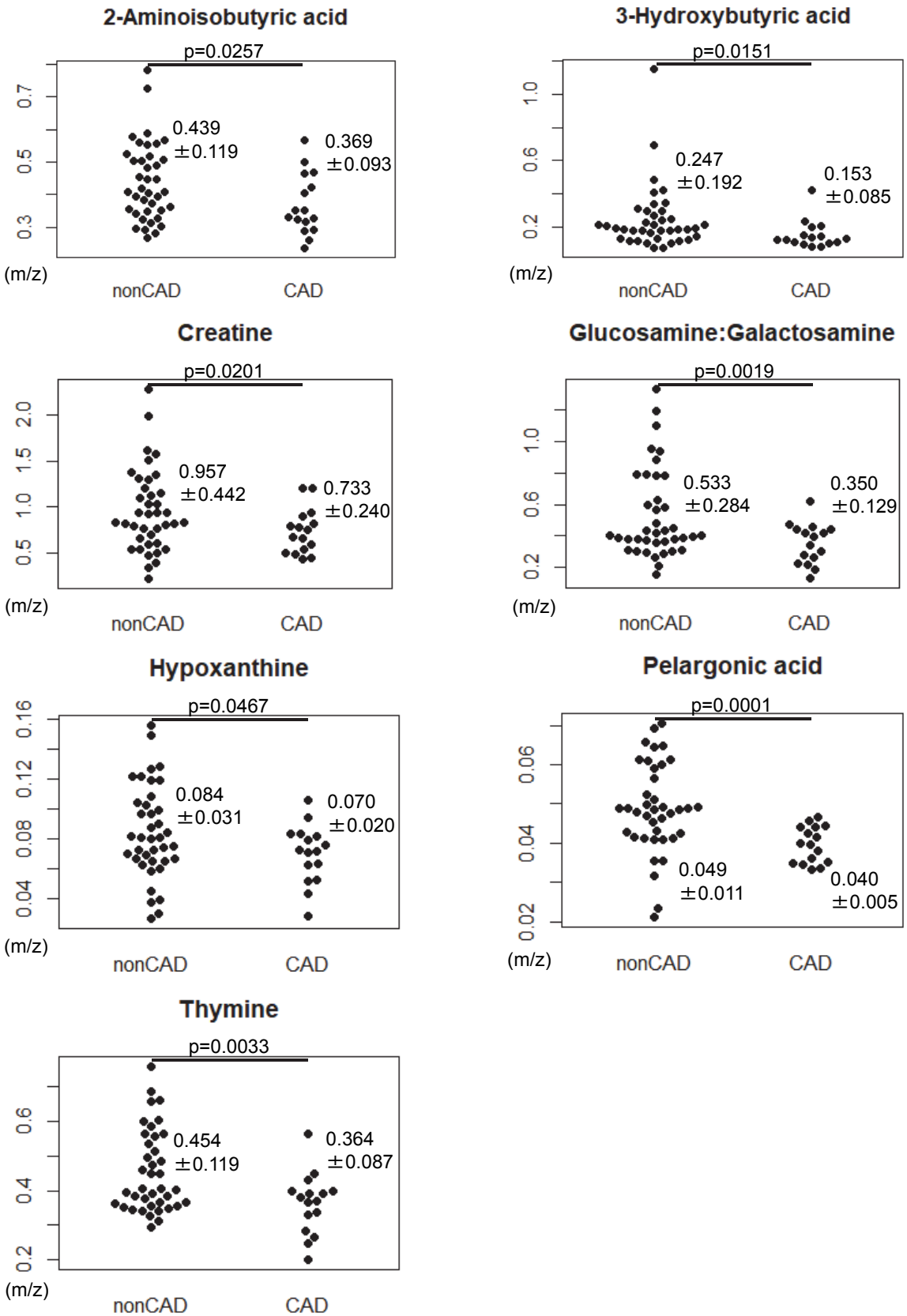

Fig. 1. Difference in metabolites statistically associated with the onset of CAD. *p* value from Welch's *t*-test comparing the medians.

Table 2. The C-statistics (area under the ROC curve) of each metabolite in prediction of CAD.

metabolite	AUC	95% confidence interval	
		Lower limit	Upper limit
Pelargonic acid	0.801	0.697	0.915
Glucosamine:galactosamine	0.668	0.512	0.825
Thymine	0.699	0.550	0.847
3-Hydroxybutyric acid	0.716	0.567	0.866
Creatine	0.662	0.515	0.809
2-Aminoisobutyric acid	0.683	0.528	0.837
Hypoxanthine	0.631	0.482	0.781

ROC, receiver operator characteristics

be used as an energy source when usage of glucose is impaired. Recently, the EMPA-REG OUTCOME study showed empagliflozin (a sodium-glucose cotransporter 2 inhibitor) improved cardiovascular mortality and hospitalization for heart failure[25]. It is considered that one of the possible reasons for this beneficial effect of empagliflozin is due to a function of 3-hydroxybutyric acid as alternative fuel for the energy metabolism of cells[26, 27]. Furthermore, 3-hydroxybutyric acid may suppress vascular inflammation leading to atherosclerosis. The increased expression of NOD-like receptor family, pyrin domain-containing 3 inflammasome components (NLRP3) and apoptosis-associated speck-like protein (ASC), in monocytes has been found to be one of the mechanisms that links T2DM and vascular inflammation[28-30]. Interestingly, a recent study has shown that 3-hydroxybutyric acid reduces NLRP3 inflammasome-mediated interleukin (IL)-1β and IL-18 production in human monocytes[31]. Creatine also functions as a component in the cellular energy shuttle system. A previous meta-analysis has shown that phosphocreatine administration in patients with acute or chronic heart disease may reduce all-cause short-term mortality and improve left ventricular ejection fraction[32]. Other studies also reported that creatine can reduce myocardial stunning[33] or infarct size of brain[34] in ischemic mouse model. However, the direct effect of both of these two metabolites on atherosclerosis has not been evaluated, and it is not clear whether these can reduce the onset of CAD.

Thymine is a pyrimidine nucleobase, and 2-aminoisobutyric acid is an end-product of pyrimidine metabolism. Hypoxanthine is a purine derivative and a reaction intermediate in the metabolism of adenosine and in the formation of nuclei acids by the salvage pathway. Among these substrates related to nucleic acid and its metabolism, hypoxanthine has been reported to be associated with atherosclerosis. It has been reported that hypoxanthine increased serum cholesterol levels and the atherosclerotic plaque area with increasing reactive oxygen species (ROS) production in ApoE-knockout mice[35]. Furthermore, it has been also reported that hypoxanthine aggravated myocardial and renal graft ischemia/reperfusion injury by ROS[36, 37]. Although these results seem to be inconsistent with our data, the direct impact of hypoxanthine on atherosclerosis and detailed mechanism are still unclear.

Pelargonic acid is a nine-carbon, chained, monocarboxylic acid that occurs in epidermis and blood in humans, and is known as a substance associated with body odor. Although the levels of this metabolite were associated with the future onset of CAD in our study, little is known regarding the association of these metabolites with atherosclerotic disease.

Interestingly, Suhre K *et al.* reported that a multiplatform metabolomics study showed increased serum 3-hydroxybutyric acid level, decreased pelargonic acid level, and unchanged creatine and hypoxanthine levels in type 2 diabetic patients compared with healthy subjects[38]. Our findings and theirs suggest the possibility that pelargonic acid is related to the onset of CAD under diabetic conditions. On the other hand, 3-hydroxybutyric acid, creatine and hypoxanthine may be associated with CAD independent of the presence of type 2 diabetes. These hypotheses need to be verified by a further clinical intervention study with large sample size. Regarding 2-aminoisobutyric acid, glucosamine and thymine, no reports have compared serum levels between patients with DM and non-diabetic subjects and, thus, further investigations would be necessary to clarify the associations between these metabolites and type 2 diabetes.

Our study has further several limitations. First, the number of the study subjects was too small to lead a robust conclusion. Furthermore, we performed multiple statistical analyses in these subjects, which would generate false-positive results derived from multiple testing. If strict Bonferroni's multiple comparison pro-

cedure was applied for statistical tests to control the family-wise type 1 error to <0.05, the corrected level of significance was supposed to be $5.2*10^{-4}$ [$=0.05/96$]. This correction revealed that only pelargonic acid remained to be significantly associated with CAD. Second, although FRS, HbA1c, and diabetes duration was matched between subjects having experienced CAD and control subjects, the small sample size in our pilot study does not allow adjustment for other clinical variables. Therefore, we could not exclude the possibility that our results were influenced by the clinical background. Similarly, since the number of subjects with CAD was small, the CAD group included subjects who had CAD history at baseline and we could not analyze the data in this regard. Further studies with large sample size would be necessary to confirm our findings. Third, the serum samples used for metabolome analysis had been in storage for about 10 years before the analysis. The long-term stability of the metabolites in serum stored at −80℃ has not been evaluated. Moreover, some metabolites could change in the process of serum generation, because platelets release proteins and metabolites into the serum during clotting at room temperature according to previous reports[39-41]. On the other hand, another study showed that serum samples are more suitable for biomarker detection, because of good reproducibility of serum metabolite measurements and generally higher concentrations of the metabolites in serum than in plasma[42]. Anyway, it was reported that there were some differences in metabolite concentrations between serum and plasma, while these profiles were highly correlated. Our pilot study using samples taken from normal subjects also revealed similar results: there were differences in absolute concentration between the two matrices, while overall correlation was high (data not shown). Thus, to increase our confidence, our findings should be supported by further testing under different sample conditions such as short-term storage or plasma. Fourth, we could not draw any conclusion from the present study as to whether there are direct relationships between the detected metabolites and atherosclerosis. Future studies using *in vivo* or *in vitro* models will be necessary to elucidate this point. Finally, although CE-MS analysis is able to evaluate global ionogenic compounds simultaneously, single analysis cannot fully cover the comprehensive metabolic profile of a biological sample. A combination with other MS analysis would enable investigation of metabolomic signatures with broader coverage. Notwithstanding these limitations, our study indicates that difference of specific metabolites could be associated with future onset of CAD in patients with T2DM.

In conclusion, we identified 7 metabolites related to long-term future onset of CAD in Japanese patients with T2DM using current technology, CE-TOFMS.

Declarations

Ethics Approval and Consent to Participate

The Research Ethics Committee of Osaka University Graduate School of Medicine approved the study protocol 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 Publications

Not applicable

Availability of Data and Materials

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

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 the contents of the manuscript and editorial decisions. All the authors contributed to the study design and were involved at all the stages of manuscript development. KO and NK drafted the manuscript. All the 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 and has full access to all the data and takes responsibility for the integrity of the data and accuracy of data analysis.

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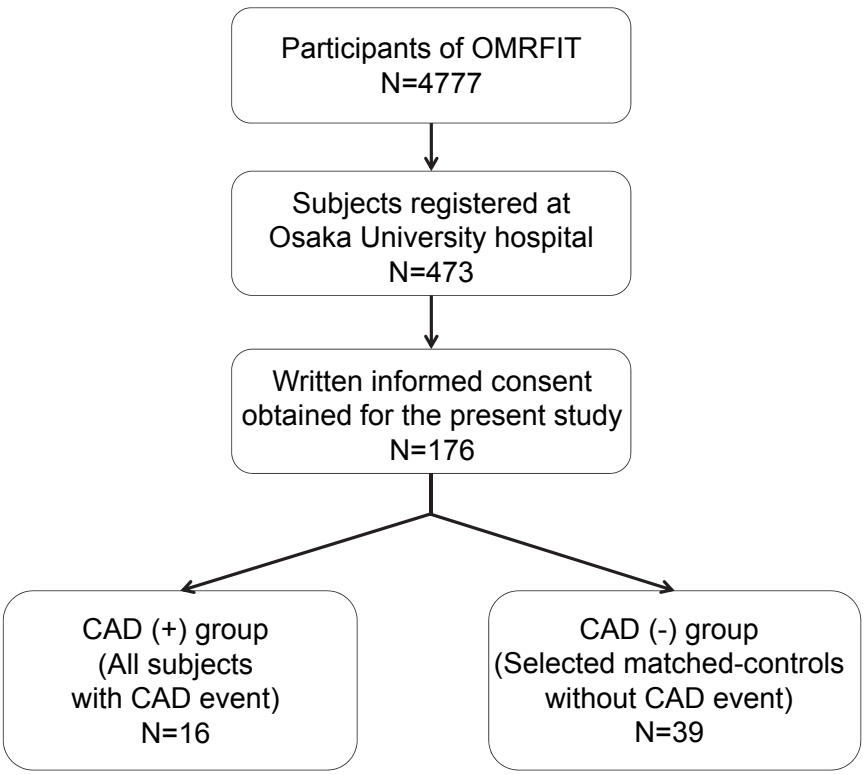


Supplementary Fig. 1. Disposition of study subjects

In the present study, the subjects were recruited from 473 subjects who were enrolled in the Order-Made multiple Risk Factor Investigation Trial (OMRFIT) at Osaka University Hospital. Among the 176 type 2 diabetic subjects who were enrolled in the present study, 16 subjects who developed CAD events during the observation period were selected as the CAD group. From the 160 subjects without CAD, 39 control subjects who were matched to the CAD group for Framingham Coronary Heart Disease Risk Score, diabetes duration, and HbA1c were selected as the non-CAD group.

Supplementary Table 1. A list of detected metabolites with known identity by CE-MS analysis in at least 75% of subjects in each group, with and without CAD.

Metabolite	Non-CAD group (n=39)	CAD group (n=16)	p value[1]
Urea	27.823±7.339	32.306±17.695	0.341
Ethanolamine	0.101±0.034	0.112±0.052	0.419
Glycine	0.350±0.129	2.487±0.883	0.101
Trimethylamine N-oxide	0.225±0.216	0.342±0.307	0.182
Sarcosine	0.033±0.014	0.028±0.010	0.219
β-Alanine	0.038±0.026	0.034±0.011	0.409
Alanine	5.630±1.432	6.275±1.693	0.193
Glycerol	4.036±1.013	4.266±0.915	0.418
2-Aminoisobutyric acid	0.439±0.119	0.370±0.093	0.026
3-Aminoisobutyric acid	0.040±0.044	0.052±0.039	0.334
GABA	0.016±0.010	0.017±0.009	0.545
Choline	2.565±2.007	3.261±2.442	0.323
Serine	1.635±0.721	1.916±0.932	0.291
Creatinine	1.168±0.384	1.943±2.236	0.188
Proline	4.667±1.162	4.801±1.786	0.786
Guanidoacetic acid	0.049±0.016	0.053±0.020	0.497
Valine	6.692±1.479	6.768±1.910	0.889
Betaine	1.330±0.288	1.421±0.387	0.411
Threonine	1.756±0.562	1.874±0.867	0.623
Taurine	0.182±0.067	0.182±0.055	0.990
Imidazole-4-acetic acid	0.006±0.002	0.006±0.002	0.600
Thymine	0.454±0.119	0.364±0.087	0.003
Pipecolic acid	0.052±0.057	0.039±0.013	0.165
Hydroxyproline	0.151±0.051	0.163±0.089	0.632
Creatine	0.957±0.442	0.734±0.240	0.020
Isoleucine	3.098±0.869	2.932±1.096	0.594
Leucine	6.638±3.916	7.794±5.032	0.419
Asparagine	0.169±0.116	0.220±0.155	0.249
Ornithine	1.087±0.331	1.132±0.406	0.702
Aspartic acid	0.907±0.818	1.252±0.997	0.232
Hypoxanthine	0.084±0.031	0.070±0.020	0.047
Trigonelline	0.040±0.049	0.043±0.036	0.792
1H-Imidazole-4-propiolic acid	0.011±0.008	0.026±0.055	0.298
Stachydrine	0.314±0.294	0.477±0.529	0.261
4-Guanidinobutyric acid	0.023±0.010	0.023±0.011	0.839
γ-Butyrobetaine	0.050±0.012	0.061±0.025	0.126
Glutamine	0.411±0.406	0.567±0.495	0.275
Lysine	3.263±1.532	3.357±1656	0.847
Glutamic acid	6.466±1.503	6.821±2.192	0.560
Histidine	0.867±0.442	1.091±0.724	0.265

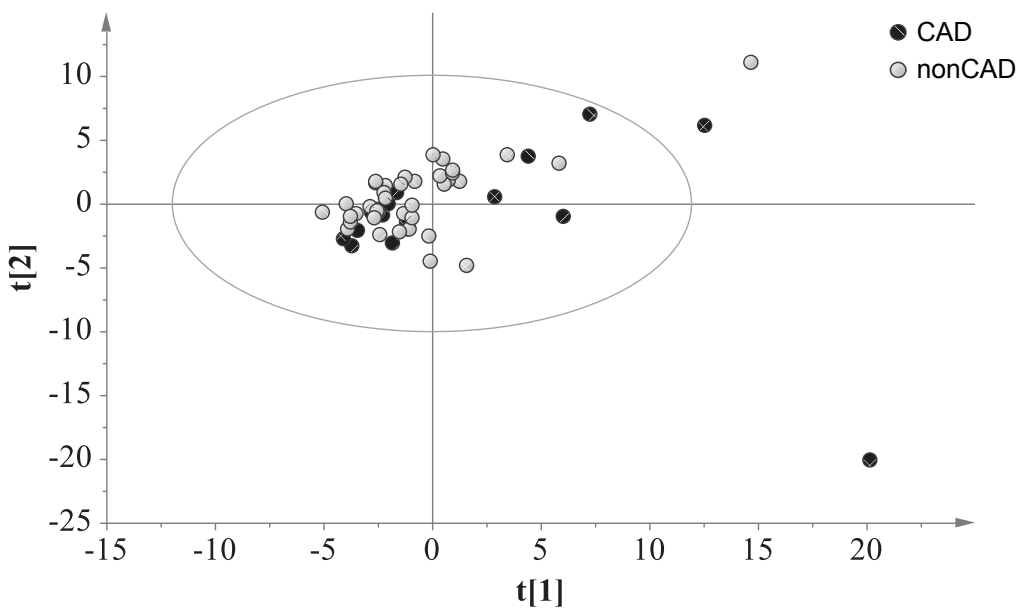
(Cont. Supplementary Table 1)

Metabolite	Non-CAD group (n = 39)	CAD group (n = 16)	p value[1]
N6-Methyllsine	0.070 ± 0.044	0.062 ± 0.021	0.385
Carnitine	1.517 ± 0.255	1.549 ± 0.278	0.693
Methionine sulfoxide	0.339 ± 0.166	0.333 ± 0.168	0.903
Phenylalanine	2.526 ± 1.351	3.106 ± 1.768	0.251
1-methylhistidine; 3-methlhistidine	0.167 ± 0.158	0.245 ± 0.286	0.317
N5-Ethlglutamine	0.017 ± 0.007	0.022 ± 0.008	0.073
N-Acetylornithine	0.015 ± 0.013	0.016 ± 0.013	0.806
Arginine	3.169 ± 1.470	3.927 ± 2.470	0.266
Guanidinosuccinic acid	0.005 ± 0.005	0.016 ± 0.042	0.303
Citrulline	0.356 ± 0.127	0.447 ± 0.257	0.194
Galactosamine; Glucosamine	0.533 ± 0.284	0.350 ± 0.129	0.002
Paraxanthine	0.037 ± 0.019	0.033 ± 0.026	0.567
Tyrosine	1.149 ± 0.597	1.206 ± 0.728	0.782
SDMA	0.012 ± 0.006	0.018 ± 0.018	0.233
Tryptophan	0.822 ± 0.193	0.800 ± 0.238	0.749
Kynurenine	0.025 ± 0.007	0.029 ± 0.010	0.127
Cystine	0.023 ± 0.012	0.024 ± 0.010	0.882
Uridine	0.045 ± 0.012	0.055 ± 0.034	0.259
Glycerophosphocholine	0.109 ± 0.026	0.111 ± 0.027	0.821
Isobutyric acid; Butyric acid	0.043 ± 0.009	0.042 ± 0.009	0.725
Lactic acid	8.670 ± 2.617	9.226 ± 2.365	0.449
Isovaleric acid; valeric acid	0.266 ± 0.079	0.226 ± 0.066	0.063
3-Hydroxybutyric acid	0.247 ± 0.192	0.153 ± 0.085	0.015
2-Hydroxybutyric acid	0.264 ± 0.087	0.216 ± 0.083	0.065
Glyceric acid	0.127 ± 0.043	0.120 ± 0.029	0.505
Hexanoic acid	0.048 ± 0.013	0.046 ± 0.012	0.540
Succinic acid	0.085 ± 0.017	0.083 ± 0.017	0.650
2-Hydroxyvaleleric acid	0.063 ± 0.034	0.060 ± 0.028	0.728
Isethionic acid	0.006 ± 0.003	0.011 ± 0.012	0.190
5-Oxoproline	0.467 ± 0.188	0.545 ± 0.187	0.169
2-Hydroxy-4-methylvaleric acid	0.010 ± 0.003	0.010 ± 0.002	0.963
Malic acid	0.102 ± 0.039	0.096 ± 0.029	0.514
Threonic acid	0.098 ± 0.024	0.126 ± 0.093	0.248
Octanoic acid	0.020 ± 0.009	0.018 ± 0.007	0.403
2-Hydroxyglutaric acid	0.042 ± 0.021	0.040 ± 0.014	0.666
Citramalic acid	0.032 ± 0.026	0.034 ± 0.015	0.762
Pelargonic acid	0.049 ± 0.011	0.040 ± 0.005	0.00009
Uric acid	0.050 ± 0.140	0.982 ± 0.280	0.089
Glycerol 3-phosphate	0.006 ± 0.002	0.006 ± 0.002	0.922
Decanoic acid	0.039 ± 0.013	0.033 ± 0.010	0.075

(Cont. Supplementary Table 1)

Metabolite	Non-CAD group (n=39)	CAD group (n=16)	p value[1]
cis-Aconitic acid	0.033±0.014	0.037±0.011	0.251
Hippuric acid	0.024±0.028	0.053±0.088	0.210
Isocitric acid	0.025±0.009	0.029±0.015	0.452
Citric acid	0.613±0.094	0.627±0.177	0.766
Quinic acid	0.017±0.016	0.013±0.011	0.322
Gluconic acid	0.034±0.011	0.057±0.079	0.266
Lauric acid	0.206±0.035	0.214±0.020	0.326
3-Indoxylsulfuric acid	0.045±0.035	0.020±0.547	0.268
Myristic acid	0.010±0.004	0.009±0.005	0.663
Ascorbate 2-sulfate	0.013±0.005	0.014±0.004	0.617
N2-Phenylacetylglutamine	0.019±0.017	0.095±0.268	0.273
N-acetylneuraminic acid	0.007±0.008	0.004±0.002	0.119
Prostaglandin E2	0.065±0.017	0.063±0.020	0.759
Prostaglandin F2α	0.027±0.010	0.028±0.011	0.780
Cholic acid	0.007±0.012	0.005±0.006	0.398
Glycocholic acid	0.006±0.005	0.009±0.012	0.198

[1]p value from Welch's t-test comparing the medians.



Supplementary Fig. 2. Score plots of unsupervised PCA of all diabetic subjects with and without the onset of CAD.

To check whether groups could be differentiated according to the metabolite profile, unsupervised PCA was performed. However, it did not reveal two distinct clusters of individuals: CAD subjects (filled circles, n=16) and non-CAD subjects (open circles, n=39).