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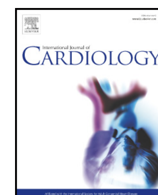

Dysregulated fatty acid metabolism in coronary ectasia: An extended lipidomic analysis

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ABSTRACT

Background: Coronary artery ectasia (CAE) is not an uncommon clinical condition, which could be associated with adverse outcome. The exact pathophysiology of the disease is poorly understood and is commonly interpreted as a variant of atherosclerosis. In this study, we sought to undertake lipidomic profiling of a group of CAE patients in an attempt to achieve better understanding of its disturbed metabolism.
Methods: Untargeted lipid profiling and complementary modelling strategies were employed to compare serum samples from 16 patients with CAE (mean age 63.5 ± 10.1 years, 6 female) and 26 controls with normal smooth coronary arteries (mean age 59.2 ± 6.6 years and 7 female). Sample preparation, LC-MS analysis and metabolite identification were performed at the Swedish Metabolomics Centre, Umeå, Sweden.
Results: Phosphatidylcholine levels were significantly distorted in the CAE patients ($p = 0.001$–0.04). Specifically, 16-carbon fatty acyl chain phosphatidylcholines (PC) were detected in lower levels. Similarly, 11 meioties of Sphyngomyelin (SM) species were detected at lower concentrations ($p = 0.000001$–0.01) in the same group. However, only three metabolites were significantly higher in the pure CAE subgroup (6 patients) when compared with the 10 mixed CAE patients (two meioties of SM species and one of PC). Atherosclerosis risk factors were not different between groups.
Conclusion: This is the first lipid profiling study reported in coronary artery ectasia. While the lower concentration and dysregulation of sphyngomyelin suggests an evidence for premature apoptosis, that of phosphatidylcholines suggests perturbed fatty acid elongation/desaturation, thus may be indicative of non-atherogenic process in CAE.
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1. Introduction

Coronary artery ectasia (CAE) is defined as abnormal dilatation of the coronary arteries, more than one and half times the diameter of either the nearest artery or distal part of the same artery. The common association with atherosclerosis is overrated mostly in the West [1], but the exact pathophysiological predisposing disturbances to ectasia remain uncertain [1,2].

The concept of metabolic profiling using gas chromatography was introduced by Horning in the early 70s [3] with the untargeted metabolites profile employed to give broad pathognomonic explanations to certain diseases. However, metabolites extraction and analysis could be quite challenging as it can induce systematic variation in composition. [4] Recent advances in metabolomics analysis generate complex stream of data, which requires appropriate chemometrics e.g. principal component analysis (PCA), partial least squares of latent structures (PLS), and orthogonal PLS (OPLS) in order to validate the biological data.

With the current uncertainty about the exact pathomechanisms of CAE we sought to adopt metabolic profiling techniques in investigating a group of patients with CAE in an attempt to achieve better understanding of the condition. This is the first study using detailed personalized metabolites profiling technology in CAE blood samples and comparing them with non-ectatic arteries.

2. Methods

2.1. Patients selection

Over 18,000 angiogram from Umeå Heart Centre of the Norrlands University Hospital, Sweden and Letterkenny general hospital, Ireland, were examined for an evidence of CAE, defined as an enlargement of the coronary diameter >1.5 that of the rest of the artery or adjacent artery diameter [2]. All patients had undergone a coronary angiogram because of typical or atypical chest discomfort suggestive of angina. Two independent cardiologists reviewed all identified angiograms and the diagnosis of CAE was confirmed. The pattern of CAE stratified the patients into two groups, pure ectasia with no evidence for atherosclerotic disease in the form of plaque formation, wall irregularities, narrowing or obstruction

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in any of the territories of the coronary tree…etc. and those with mixed disease who have CAE and evidence for atherosclerosis.

Cardiovascular risk factors for atherosclerotic coronary artery disease were collected from the patients' medical notes and control group at the time of presentation. This data included hypertension, diabetes mellitus, smoking or ex-smoking, family history of coronary heart disease and hyperlipidaemia. Both centres used standard definitions for risk factors. Hypertension was defined according to the JNC-7 guidelines as systolic blood pressure ≥ 140/90 mm Hg, diastolic blood pressure ≥ 90 mm Hg and/or use of antihypertensive medications. Diabetes was defined as overnight fasting blood glucose ≥ 7 mmol/l (126 mg/dl), postprandial blood glucose ≥11 mmol/l (200 mg/dl) or use of insulin or oral hypoglycaemic agents. Body mass index (BMI) was calculated using height and weight measurements. Blood lipids were measured using standard enzymatic methods. Hypercholesterolemia was defined as total cholesterol ≥6.2 mmol/l (240 mg/dl), low-density lipoprotein cholesterol ≥4.14 mmol/l (160 mg/dl) or use of lipid-lowering medications. However, all patients in both groups were on statins at the time of sample collections. Family history of premature CAD was noted if a male first-degree relative developed CAD aged <55 or a female first-degree relative aged <65. Patients were classified smokers if they had smoked during the last month. [5].

A research nurse contacted the patients by phone and explained to them the project details. This was followed by sending all patients a detailed information sheet, approved by the Ethics Committee, and requesting them to sign a consent form. After obtaining the consent, the nurse visited those patients at the nearest health centre where she collected blood samples from 16 patients (age 39–69 years), who agreed to give blood, following the conventional recommendations. A control group of 70 patients (48–83 years of age) with no evidence of coronary stenosis or even luminal irregularities on conventional angiography underwent similar protocol. They had coronary angiography because of atypical chest pain, which proved to be non-cardiac, having demonstrated normal coronary arteries. Controls too had consented to have blood samples taken at the Umeå Heart Centre as a part of another study [Calcific Coronary Artery Disease (CCAD)] established at the Heart Centre in 2008. Patients and controls blood was withdrawn through a venipuncture in the Cubital fossa region. The separated serum was transferred in an Eppendorf tube and stored at −80 °C until taken out for metabolomics profiling.

This study was approved by the Regional Ethics Committee in Umeå (Sweden) and Letterkenny general hospital (North west health service executive, Ireland).

2.2. Metabolomics blood analysis

Blood analysis was performed using conventional metabolomics protocol applied at the Swedish Metabolomics Centre, Umeå. The set of samples was first analyzed in the positive mode. After all samples had been analyzed, the instrument was switched to the negative mode and a second injection of each sample was performed.

2.3. Plasma lipid extraction protocol

Plasma extraction: The plasma was extracted using a mixer mill and all extraction steps in Eppendorf tubes as follows [6].

An aliquot of 100 μl of plasma was mixed with 900 μl of MeOH: H2O (9:1) containing methanol-soluble internal standards in an Eppendorf tube. Tubes were incubated in ice for 10 min, then mixer beads were added and the tubes were shaken in a mixer for 2 min (30 Hz). The beads were later removed and the tubes incubated in ice for a further 2 h before the samples were centrifuged for 5 min at 13,000 RPM in an Eppendorf centrifuge. A 200 μl of the organic layer was then transferred to a glass vial and was left to evaporate overnight in a fume hood. Extracts were stored at −40 °C until analysis.

2.4. Chromatographic separation

Chromatographic separation was performed using Agilent 1290 Infinity UHPLC-system (Agilent Technologies, Waldbronn, Germany). One μl of extracted plasma sample were injected onto an Acquity UPLC CSH, 2.1 × 100 mm, 1.7 μm C_{18} column in combination with a 2.1 mm × 5 mm, 1.7 μm VanGuard precolumn (Waters Corporation, Milford, MA, USA) held at 60 °C. The gradient elution buffers were A (60:40 acetonitrile: water, 10 mM ammonium formate, 0.1% formic acid) and B (90:10 2-propanol: acetonitrile, 10 mM ammonium formate, 0.1% formic acid), and the flow-rate was 0.5 ml min^{-1}.

Table 1
This table depicts the demographics and cardiovascular risk factors in coronary ectasia group (CAE) and controls.

	Ectasia group ($N = 16$)	Control ($N = 26$)	p value
Gender (female, %)	(6, 38%)	(7, 27%)	N/A
Age (mean ± SD) years	63.5 ± 10.1	59.2 ± 6.6	NS
Hypertension (N, %)	10, 62%	17, 65%	NS
Diabetes mellitus (N, %)	(4, 25%)	(4, 15%)	NS
Hyperlipidaemia (N, %)	9, 56%	16, 54%	NS
BMI (mean ± SD)	25.8 ± 4.5	27.1 ± 3.9	NS
Family history of IHD (N, %)	7, 38%	(12, 46%)	NS
Smoking (N, %)	7, 43%	12, 46%	NS

Table 2
Statistics obtained for the models of CAE vs control in positive and in negative modes, indicating the number of OPLS-DA components, variance of X and Y explained by the model (R2X, R2Y), variance of Y predicted by the model (Q2), CV-ANOVA p-value and permutation test p-value.

Group comparison Mode	CAE vs control Negative	CAE vscontrol Positive
No. of components	1 + 0	1 + 0
R2X (cum)	0.247	0.163
R2X (cum)	0.379	0.388
Q2Y (cum)	0.172	0.180
CV-ANOVA (p-value)	0.027	0.030
Permutation testing (p-value)	0.006	0.008

2.5. UPLC-MS analysis

The compounds were eluted with a linear gradient using an initial condition 15% B, and increased to 30% B at 2.4 min, 48% at 3 min, 60% B at 10 min, 82% at 13.2 min and 99% B at 14.0 min, where it was held at 99% for 2.4 min. B was then decreased to initial condition 15% over 0.8 min where it was held for 2.8 min to equilibrate the column. An additional wash of the injection valve, with 99% B and flow-rate of 5.0 ml min^{-1} for 0.3 min, were performed before the next injection.

The compounds were detected with an Agilent 6550 Q-TOF mass spectrometer equipped with a jet stream electrospray ion source operating in positive or negative ion mode. The settings were kept identical between the modes, with the exception of the capillary voltage. A reference interface was connected for accurate mass measurements; the reference ions purine (2 μM) and HP-0921 (Hexakis, 1H, 1H, 3H-tetrafluoropropoxy, phosphazine) (2.5 μM) [Agilent Technologies, Santa Clara, CA, USA] were infused directly into the MS at a flow rate of 0.07 ml min^{-1} for internal calibration, and the monitored ions were purine m/z 121.05087 and m/z 119.03632; HP-0921 m/z 922.00980 and m/z 966.000725 for positive and negative modes, respectively.

The gas temperature was set to 150 °C, the drying gas flow to 12 l min^{-1} and the nebulizer pressure 35 psig. The sheath gas temperature was set to 350 °C and the sheath gas flow 11 l min^{-1}. The capillary voltage was set to 3500 V in the positive ion mode, and to 3500 V in the negative ion mode. The nozzle voltage was 1000 V; the fragmentor voltage was 380 V, the skimmer 45 V and the OCT 1 RF Vpp 750 V. The collision energy was set to 0 V and the m/z range was 60–1700. The data was collected in centroid mode with an acquisition rate of 4 scans s^{-1} (1977 transients/spectrum). The diode array detector was set to scan the interval 190–640 nm with a step length of 2 nm and a slit width of 4 nm.

2.6. Identification of statistically significant signals detected by UPLC-MS

Mass Feature Extraction (MFE) from the data acquired was performed using the MassHunter™ Qualitative Analysis software package, version B06.00 (Agilent Technologies Inc., Santa Clara, CA, USA). Extracted features were aligned and matched between samples using Mass Profiler Professional™ 12.5 (Agilent Technologies Inc., Santa Clara, CA, USA). In-house database with exact mass and experimental retention times of lipids were used for identification.

2.7. Statistical analysis

Univariate t-tests at the 95% confidence level considering equal variances were performed, as well as Mann–Whitney U tests. Benjamini-Hochberg correction with $\alpha = 0.05$ was used and U test to control the False Discovery Rate [7]. All calculations were performed in MATLAB® (MathWorks®, Natick, USA).

Principal Components Analysis (PCA) is an unsupervised multivariate method that is used for data overview and to detect trends and outliers. The principal components represent the directions of largest variance in the data, approximating the data in the least squares sense. It allows simpler visualization by reducing data dimensionality, and separating information from random variation. [8].

Projection to Latent Structures (PLS) is a (supervised) multivariate regression method related to PCA. Orthogonal Partial Least Squares (OPLS), a modification of the PLS method, finds the relations between two matrices (data X and response Y), by maximizing the covariance of their latent variables. It allows identification of variables more correlated to a response. It also provides better interpretation of the relevant variables than PLS, by decomposing the data in "predictive" information related to the response Y (as concentrations, classes), "orthogonal" structured information not related to the response (as instrumental, biological variations), and residual variation. [9].

PLS-DA (Discriminant Analysis) is a variant of the previous (O) PLS methods, used when Y is categorical, as when discriminating between different classes. [10] Statistical significance of metabolites and features was considered if the OPLS-DA loadings p was larger than its confidence interval (the confidence interval does not cross zero), and simultaneously the t-test and Mann–Whitney U test had p-values < 0.05 after Benjamini-Hochberg correction [7,11].

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