International Journal of Cardiology 197 (2015) 192–199

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Perturbations in fatty acid metabolism and apoptosis are manifested in calcific coronary artery disease: An exploratory lipidomic study☆

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ARTICLE INFO

Article history:
Received 15 March 2015
Received in revised form 22 May 2015
Accepted 18 June 2015
Available online 23 June 2015

Keywords:
Lipidomics
Metabonomics
Metabolomics
Calcification
Fatty acid
Apoptosis

ABSTRACT

Background: Controversy exists concerning the beneficial or harmful effects of the presence of ectopic calcification in the coronary arteries. Additionally, further elucidation of the exact pathophysiological mechanism is needed. In this study, we sought to identify metabolic markers of vascular calcification that could assist in understanding the disease, monitoring its progress and generating hypotheses describing its pathophysiology.
Methods: Untargeted lipid profiling and complementary modeling strategies were employed to compare serum samples from patients with different levels of calcific coronary artery disease (CCAD) based on their calcium score (CS). Subsequently, patients were divided into three groups: no calcification (NC; CS = 0; n = 26), mild calcification (MC; CS:1–250; n = 27) and severe (SC; CS > 250; n = 17).
Results: Phosphatidylcholine levels were found to be significantly altered in the disease states (p = 0.001–0.04). Specifically, 18-carbon fatty acyl chain (FAC) phosphatidylcholines were detected in lower levels in the SC group, while 20:4 FAC lipid species were detected in higher concentrations. A statistical trend was observed with phosphatidylcholine lipids in the MC group, showing the same tendency as with the SC group. We also observed several sphingomyelin signals present at lower intensities in SC when compared with NC or MC groups (p = 0.000001–0.01).
Conclusions: This is the first lipid profiling study reported in CCAD. Our data demonstrate dysregulations of phosphatidylcholine lipid species, which suggest perturbations in fatty acid elongation/desaturation. The altered levels of the 18-carbon and 20:4 FAC lipids may be indicative of disturbed inflammation homeostasis. The marked sphingomyelin dysregulation in SC is consistent with profound apoptosis as a potential mechanism of CCAD.

1. Introduction

Vascular calcification is a pathological condition of ectopic bone formation with controversial etiology [1], that has been associated with conventional cardiovascular risk factors e.g. age, male gender, white race/ethnicity, hypertension, body mass index, diabetes mellitus and family history of myocardial infarction [2,3]. While calcification can remain silent for decades, it may present with intractable symptoms unresponsive to conventional interventional treatments irrespective of the presence of arterial obstructive lesions [4]. Moreover, the exact risk/benefit relationship of the presence of vascular calcification and acute coronary events remain, to an extent, unclear with some evidence showing increased risk [5,6], while others have demonstrated only recurrent stable angina [7,8] with many patients having extensive calcification but good prognosis [9,10]. This conflicting evidence — yet the ability of coronary calcification for predicting cardiovascular events — highlights the need for identifying diagnostic biomarkers that could assist in disease detection, stratification and monitoring.

Coronary calcification can be part of the atherosclerosis pathology, however, it can also appear before luminal narrowing, or can be primarily diffuse with no evidence for sizable plaque formation. The natural course of coronary calcification is typically progression even in patients with long term statin therapy [4]. This raises the possibility of potential independent mechanisms for calcification other than simply the conventional atherosclerosis cascade [11]. Currently, no clear pathway dysregulation mechanism is known [12]. These characteristics of vascular calcification demonstrate the need for: 1) elucidating the dysregulated biological pathways specific for cardiovascular calcification and 2) highlighting potential biological patterns in the course of coronary calcification progression.

☆ Vorkas PA et al.; lipid dysregulations in coronary calcification.
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http://dx.doi.org/10.1016/j.ijcard.2015.06.048
0167-5273/

A relatively novel technology that is suitable for addressing both biomarker discovery and biological pathway elucidation is metabolic profiling (metabonomics) [13,14]. Metabolic profiling enables holistic analysis and rationalization of systemic fluctuations of small molecules in a biological system [15–17]. Lipid profiling (lipidomics) is a subcategory of metabolic profiling, focusing on the lipophilic molecules in a biological matrix (lipids) [18,19]. Lipids have been proven to be more than energy storage molecules or cell membrane building blocks; they have been shown to be involved in signaling and disease [20–22]. Most importantly, lipid diversity far exceeds the expected number of genes responsible for their metabolism, demonstrating influences from environmental factors [23]. In the context of vascular calcification, the specific lipid classes and lipid mechanisms involved are still not completely understood [12].

The aim of this study was to apply a lipid profiling approach, specifically an untargeted method employing ultra performance liquid chromatography coupled to mass spectrometry (UPLC–MS) [19], to profile the serum lipidome of a cohort of symptomatic angina patients presenting with various degrees of coronary calcification, but no flow-limiting lesions on conventional angiography. We compared patient serum metabolic profiles with those from individuals without detectable coronary calcification. The ultimate objectives of this study were 1) to identify potential/candidate diagnostic biomarkers for CCAD, in an easily accessible biological matrix and 2) to generate rational hypotheses that can explain the underlying calcification pathophysiology, based on the metabolic findings. To the best of our knowledge, this is the first metabolic profiling application with regard to calcific cardiovascular disease, in any biological matrix (blood, tissue or cells).

2. Methods

2.1. Patients

Serum samples were collected from 70 patients (48–83 years of age) who visited the clinic (Heart Centre and Department of Public Health and Clinical Medicine, Umea University, Umea, Sweden) with exertional angina. No patient had prior myocardial infarction or coronary artery intervention (PCI). No patient had valve disease, heart failure or renal dysfunction. Patients underwent a multislice CT scan of the chest from which their coronary calcium score (CS) was measured using the Agatston score and Hounsfield units: 26 patients had no evidence of coronary artery calcification (zero CS; controls; NC), 27 had CS between 1 and 250 (mild calcification group; MC), and 17 patients had a calcium score of >250 (severe calcification group; SC). Patients completed a clinical questionnaire investigating their lifestyle, prior clinical conditions, and medications.

A research nurse explained the program to the patients who signed an informed consent to participate in the study. Subsequently patients underwent venipuncture in the cubital fossa by an experienced nurse. The sample was left to clot for 30 min and centrifuged. The separated serum was decanted in an Eppendorf tube and stored at −80 °C. The study was approved by the Regional Ethics Committee of Umea. Patients' demographics are summarized in Table 1.

Table 1
Patients' demographics for the three patient groups included in the present study.

Group	No calcification	Mild calcification	Severe calcification	ANOVA p-Value
Number of patients	26	27	17	–
Calcium score median (range)	0 (n/a)	50 (4–219)	801 (254–1840)	–
Median age (range)[a]	61 (50–74)	67 (50–80)	67 (48–83)	0.004[a]
Male gender (%)[a]	7 (27)	11 (41)	11 (65)	0.05[a]
Median BMI (range)	26.4 (21.5–37)	26.4 (19.2–41.4)	27.8 (23.3–37.3)	0.72
Median of blood pressure, mm Hg (range)				
Systolic	138 (110–180)	140 (104–172)	138 (120–178)	0.77
Diastolic	84 (62–100)	80 (66–96)	82 (62–100)	0.44
Antihypertensives (%)	17 (65)	22 (81)	15 (88)	0.18
Smoker				
Yes (%)	1 (4)	3 (11)	2 (12)	0.56
Former (%)	12 (46)	13 (48)	10 (59)	0.71
Diabetes mellitus (%)	4 (15)	3 (11)	5 (29)	0.29
Osteoporosis (%)	2 (8)	2 (7)	1 (6)	0.97
Hypercholesterolemia (%)[a]	13 (50)	23 (85)	17 (100)	0.0001[a]
Statins (%)[a]	10 (38)	21 (78)	15 (88)	0.0005[a]
Aspirin (%)[a]	11 (42)	21 (78)	13 (76)	0.01[a]

CCAD: calcific coronary artery disease.
[a] These factors were further tested using multivariate statistics and were not attributed to any contribution to the disease-based models.

2.2. Lipid profiling using ultra-performance liquid chromatography coupled to mass spectrometry (UPLC–MS) analysis

2.2.1. Serum liquid–liquid extraction

An aliquot of 100 μL of serum was mixed with 600 μL of organic solvent mixture in an eppendorf tube. The organic solvent mixture consisted of dichloromethane (Chromasolv, LC–MS grade, Fluka, Germany)/methanol (Chromasolv, LC–MS grade, Fluka, Germany) (3:1). After intense vortexing for 30 s, samples were centrifuged for 10 min at 12,000 *g* and 4 °C. A volume of 500 μL of the organic layer was then transferred to a glass vial and left to evaporate overnight in a fume hood. Extracts were stored at −40 °C until analysis.

2.2.2. UPLC–MS analysis

Samples were reconstituted in 400 μL of H_2O/isopropanol/acetonitrile (1:2:1) (Optima, LC–MS grade, Fisher Scientific, USA), and transferred into total recovery vials (Waters, USA), after centrifugation for 10 min at 5000 *g* and 4 °C.

The untargeted UPLC–MS lipid profiling analysis was conducted as previously described [19]. UPLC separation was performed using an Acquity UPLC System (Waters Corporation, USA). An Acquity UPLC CSH column (C18 2.1 × 100 mm, 1.7 μm; Waters Corporation, USA) was used. Column temperature was set at 55 °C, flow rate of 0.4 mL/min. Injection volumes of 3 μL and 7 μL were used for positive and negative ionization modes respectively. Mobile phase A consisted of acetonitrile/water (60:40) and mobile phase B isopropanol/acetonitrile (90:10). In both solutions ammonium formate (LC–MS grade, Fluka, USA) was diluted to 10 mM and formic acid (MS grade, Fluka, USA) to 0.1%. The chromatographic gradient program is summarized in Table S1 of the Supplementary material. Mass spectrometric detection was achieved using a Xevo G2 QTof mass spectrometer (Waters MS Technologies, UK). Mass spectrometer parameters are summarized in Supplementary methods.

A QC (quality control) strategy [24] was used for the UPLC–MS analysis. Briefly, a pooled sample (referred to as quality control sample; QC) of the reconstituted extracts was prepared by combining 30 μL from each study sample. This sample was initially injected 10 times before the beginning of the run in order to condition the column. Then the sample was re-injected once at the beginning, after every 10 injections of samples, and at the end of the run (totaling 9 injections). A tight grouping of the QC samples in multivariate data analysis (MVDA), and a coefficient of variation percentage (CV%) of less than 30% for candidate biomarkers [25,26] formed the major quality control criteria. A flowchart illustrating the analytical conditions to be met by individual metabolites in order to be characterized as statistically significant is demonstrated in Fig. S1 of the Supplementary material.