Associations between specific plasma ceramides and severity of coronary-artery stenosis assessed by coronary angiography

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Short Title: Plasma ceramides and coronary artery disease

Word count: abstract 248; text 3,458 (*excluding* title page, abstract, acknowledgments, references and tables); n. 4 **Tables**; n. 3 **Supplementary Tables**

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ABSTRACT

Aim: Recent prospective studies have identified distinct plasma ceramides as strong predictors of major adverse cardiovascular events in patients with established or suspected coronary artery disease (CAD). Currently, it is uncertain whether higher levels of distinct plasma ceramides are also associated with greater angiographic severity of coronary-artery stenoses in this patient population.

Methods: We measured six previously identified high-risk plasma ceramide species [Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d18:1/22:0), Cer(d18:1/24:0) and Cer(d18:1/24:1)] in 167 consecutive patients with established or suspected CAD, who underwent urgent or elective coronary angiography.

Results: Approximately 77% of patients had a significant stenosis (≥50%) in one or more of the main coronary arteries, the majority of whom (~60%) had a significant stenosis in the left anterior descending (LAD) artery. Of the six measured plasma ceramides, higher levels of plasma Cer(d18:1/20:0) (adjusted-odds ratio 1.39, 95%CI 1.0-1.99), Cer(d18:1/22:0) (adjusted-odds ratio 1.57, 95%CI 1.08-2.29) and Cer(d18:1/24:0) (adjusted-odds ratio 1.59, 95%CI 1.08-2.32) were significantly associated with the presence of LAD stenosis ≥50%, after adjustment for age, sex, smoking, pre-existing CAD, hypertension, diabetes, dyslipidemia, lipid-lowering therapy, estimated glomerular filtration rate and plasma C-reactive protein levels. Almost identical results were found even after excluding patients (*n*=15) with acute ST-elevation myocardial infarction. Similar results were also found when patients were categorized according to Gensini severity score.

Conclusion: Our cross-sectional study shows for the first time that higher levels of specific plasma ceramides are independently associated with a greater severity of coronary-artery stenoses in the LAD artery in patients who had suspected or established CAD.

Keywords: coronary angiography; ceramides; coronary artery disease; risk factors

LIST OF ABBREVIATIONS

BMI, body mass index
Cer, ceramides
CAD, coronary artery disease
CKD, chronic kidney disease
CRP, C-reactive protein
e-GFR, estimated glomerular filtration rate
FDR, false discovery rate
LAD, left anterior descending artery
LCX, left circumflex artery
MDRD, Modification of Diet in Renal Disease
RCA, right coronary artery

INTRODUCTION

Ceramides are a family of complex lipid molecules composed of sphingosine and a fatty acid, and are found in high concentrations in cell membranes. Ceramides exert several biological effects through cellular proliferation, differentiation and apoptosis, thus interacting with multiple pathways potentially involved in chronic inflammation, oxidative stress and atherogenesis [1-4]. Indeed, literature on ceramide biology links these highly bioactive lipid molecules with many atherosclerotic processes, such as increased uptake of lipoproteins and accumulation of cholesterol within macrophages, impaired nitric oxide synthesis, increased production of reactive oxygen species, enhanced platelet activation, and increased expression of various proinflammatory cytokines [1,4-6].

Recent prospective studies found that distinct plasma ceramides [mostly plasma Cer(d18:1/16:0), Cer(d18:1/18:0) and Cer(d18:1/ 24:1)] are independently associated with an increased risk of major adverse cardiovascular events both in the general adult population and in patients with established coronary artery disease (CAD) or acute coronary syndrome (ACS) [7-11]. Moreover, a significant association between higher plasma Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d18:1/22:0) and Cer(d18:1/24:1) levels and presence of inducible myocardial ischemia has also been described in patients with established, or suspected, CAD undergoing stress myocardial perfusion scintigraphy [12].

That said, in this hypothesis-generating study, we supposed that there is a significant graded relationship between distinct plasma ceramides (associated with an increased risk of CAD) and the severity of coronary stenoses on angiography. Currently, however, no conclusive evidence is available for the existence of a significant association between the aforementioned specific plasma ceramides and greater severity of coronary-artery stenoses in patients with established or suspected CAD [10,13]. We believe that this topic

is clinically important because the observation of a significant, graded association between distinct plasma ceramide levels and the angiographic severity of coronary-artery stenoses could further expand our knowledge(s) about the complex biological mechanisms that contribute to the development of CAD, and could also provide new insights into the prevention and management of CAD.

Thus, the main aim of this study was to examine whether there were significant associations between circulating levels of six previously identified plasma ceramides associated with increased cardiovascular risk, and the severity of coronary-artery stenosis. Severity of coronary-artery stenosis was established by coronary angiography, which had been undertaken by the clinician in charge as part of normal clinical practice.

MATERIALS AND METHODS

Patients

We consecutively enrolled all patients who underwent elective or urgent coronary angiography for various clinical indications (*e.g.*, chest pain, dyspnea, suspected ischemic electrocardiographic alterations, or echocardiographic abnormalities) at the Cardiology Division of the "IRCCS Sacro Cuore" Hospital of Negrar between April 2017 and January 2018. Patients with a prior medical history of malignancy, decompensated cirrhosis or kidney failure were excluded from the study (*n*=21, i.e. conditions that may substantially alter plasma ceramide levels, irrespective of CAD)[1,6].

The study protocol was approved by the Institutional Review Board of the hospital, and informed consent for all tests was obtained from all the participants.

Clinical and Laboratory Data

Body mass index (BMI) was measured as kilograms divided by the square of height in meters. Patients were considered to have hypertension if their blood pressure was ≥140/90 mmHg or if they were treated with any anti-hypertensive drug. Information on smoking history and current use of medications was obtained from all patients by interviews during medical examinations.

Venous blood samples were drawn in the morning after an overnight fast. Serum creatinine (measured using a Jaffé rate blanked and compensated assay), lipids, troponin and other biochemical blood parameters were measured using standard laboratory procedures at the central Laboratory of the hospital. Plasma high sensitivity C-reactive protein (hs-CRP) concentrations were measured by using Beckman Coulter Image Immunochemistry System. Low-density lipoprotein (LDL)-cholesterol was calculated using the Friedewald's equation. Glomerular filtration rate (e-GFR_{MDRD}) was estimated using the four-variable Modification of Diet in Renal Disease (MDRD) study equation [10].

Pre-existing CAD was defined as a documented history of acute myocardial infarction, angina pectoris, or coronary revascularization procedures. The diagnosis of permanent atrial fibrillation was made on the basis of medical history (from reviewing hospital and physician charts from all patients) and standard 12-lead electrocardiograms. Pre-existing history of valvular heart diseases was confirmed by reviewing medical records of the hospital and echocardiograms. Previously known diabetes was defined as self-reported physician-diagnosed diabetes, or use of glucose-lowering medications (insulin or oral hypoglycemic drugs). Dyslipidemia was defined as an LDL-cholesterol level ≥2.6 mmol/L or use of any lipid-lowering agents. Chronic kidney disease (CKD) was defined as the presence of e-GFR_{MDRD} <60 mL/min/1.73 m^2 [14].

Plasma Ceramide Measurements

Blood samples for ceramide measurements were taken into ethylenediamine tetra-acetic acid (EDTA)-containing tubes, immediately preceding coronary angiography. Plasma was stored at -80°C until analysis. An expert laboratory technician, who was blinded to the clinical details of the participants, performed all measurements of plasma ceramides. Ceramide standards were purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). Plasma concentrations of Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/20:0, Cer(d18:1/22:0), Cer(d18:1/24:0), Cer(d18:1/24:1) were measured by liquid-liquid extraction with 2-propanol:ethyl acetate (4:1 v/v) and gradient reverse phase chromatography on an Agilent Poroshell 120 C18 column (4.6x50mm, 2.7 µm) [12,15]. Cer(d18:1/17:0) was used as internal standard. The apparatus consisted of an Agilent 1290 UHPLC (ultra-high-performance liquid chromatography) system coupled with an Agilent 6495 Triple Quadrupole liquid chromatography/mass spectrometry (LC/MS) system. Mobile phases consisted in LC-MS grade water (A), acetonitrile with 0.1% formic acid (B) and 10 mM ammonium acetate in 2-propanol (C). $[M+H]+\rightarrow 264$ MRM (multiple reaction monitoring) transition was selected to quantify each ceramide. Calibration standards (six points) were prepared each day in surrogate matrix (5% bovine serum albumin) at concentrations ranging from 1.0 to 0.031 µM/L for Cer(d18:1/16:0), Cer(d18:1/18:0) and Cer(d18:1/20:0), and from 10 to 0.31 µM/L for Cer(d18:1/22:0), Cer(d18:1/24:0) and Cer(d18:1/24:1), respectively. Linearity regression coefficients were R^2 >0.99 for all ceramides. Inter-assay and intra-assay coefficients of variation for precision and accuracy for all measured ceramides were <15% [12]. No matrix interference or carry over was observed.

Coronary Angiography and Echocardiography

A clinically indicated elective or urgent coronary angiography was performed in all patients by two experienced cardiologists, who were blinded to clinical and biochemical details of participants. The degree of determined stenosis of the left main stem, left anterior descending (LAD) artery, left circumflex artery (LCX) and right coronary artery (RCA) was recorded according to standard methods [16]. Significant CAD in each coronary artery was defined as the presence of at least 50% stenosis in one or more of the major coronary arteries [16]. The angiographic severity of coronary stenoses was also calculated using the Gensini score [17]. After the angiographic procedure, medical therapy or percutaneous coronary intervention and coronary artery bypass grafting were provided as clinically indicated. Trans-thoracic echocardiography (Vivid 7, GE Vingmed, Horten, Norway) was also performed in a subgroup of these patients (*n*=122, 73% of total) to measure left ventricular diameters, wall thickness, and mass according to standard criteria. Left ventricular end-diastolic and end-systolic volumes and ejection fraction at rest were also measured from the apical four-chamber and two-chamber views.

Statistical Analysis

The study was hypothesis-generating and aimed to describe associations between each of the six measured plasma ceramides that are associated with cardiovascular risk, and the angiographic severity of coronary stenoses. Data are expressed as means±SD, medians (inter-quartile ranges, IQR) or percentages. Multivariable linear regression analysis was used to test independent associations between each plasma ceramide (included as a continuous variable, *i.e*., for each SD increment) and the angiographic severity of coronary stenosis as the outcome measure, after adjustment for multiple cardiovascular risk factors. In particular, we performed two forced-entry multivariable linear regression models: the first model was adjusted for age and sex (model 1); and the second model was further adjusted for smoking, prior history of CAD, dyslipidemia (defined as LDL-cholesterol ≥2.6

mmol/L or use of lipid-lowering drugs), hypertension (blood pressure ≥140/90 mmHg or use of anti-hypertensive agents), pre-existing diabetes, plasma hs-CRP and e-GFR values (model 2). These covariates were selected as potential confounding factors based on their significance in univariate analyses or based on their biological plausibility. By logistic regression analysis, we also tested the association between each plasma ceramide and the presence of LAD stenosis ≥50%, after adjustment for the same list of the aforementioned covariates. Adjustment for multiplicity was also made using the Benjamini-Hochberg method, which is a linear step-up multiple testing procedure that controls the false discovery rate (FDR) [18]. Finally, the aforementioned multivariable regression models were repeated using the Gensini score, i.e. another widely used method for assessing the angiographic severity of coronary stenosis [17], and after excluding patients with acute ST-elevation myocardial infarction (*n*=15), in which assessment of coronary artery stenosis might be less accurate due to the presence of thrombus within the coronary artery. A *p*-value <0.05 was considered to be statistically significant. Statistical analyses were performed using STATA software, version 14.2 (STATA, College Station, Texas, USA).

RESULTS

Totally, 167 consecutive patients (123 men and 44 women) with suspected or established CAD were enrolled in the study. The main clinical and biochemical characteristics as well as the angiographic coronary data of these patients are shown in **Table 1** and **Table 2**. Overall, patients had a mean age of 69 years and were predominantly men and overweight. In addition, 34.1% of these patients had a prior history of CAD, 84.4% had dyslipidemia, 77.2% had hypertension, and 21% had diabetes (most of whom treated with

oral hypoglycemic agents). Among these patients, 30 (18%) patients underwent urgent coronary angiography for either acute ST-elevation myocardial infarction (n=15) or non-ST-elevation myocardial infarction (n=15), whereas 137 (82%) of them underwent elective (non-urgent) coronary angiography. Patients undergoing urgent coronary angiography were more likely to be female and current smokers. Fewer of these patients were hypertensive, and were treated with lipid-lowering or antiplatelet drugs. These patients also had higher plasma troponin and hs-CRP levels compared to those in the non-urgent angiography group. Moreover, these patients also tended to have higher plasma ceramide levels. No significant differences were found in age, pre-existing diabetes, BMI, e-GFR, and angiographic coronary data between the two patient groups (data not shown). As also shown in **Table 2**, a stenosis of at least 50% in one or more coronary arteries was found in 77.2% of the entire cohort of patients, most of whom (~60%) had a coronary stenosis ≥50% within the LAD artery.

Table 3 shows the effect of adjustment for multiple cardiovascular risk factors on the association between each plasma ceramide (expressed per 1-SD increment) and the angiographic severity of LAD stenosis. Higher plasma Cer(d18:1/20:0), Cer(d18:1/22:0) and Cer(d18:1/24:0) levels were significantly associated with a greater severity of LAD stenosis after adjustment for age, sex, smoking, prior history of CAD, hypertension, dyslipidemia, diabetes, plasma hs-CRP and e-GFR values (model 2). In the fully adjusted model, prior history of CAD was the only other variable that was independently associated with the severity of LAD stenosis. Notably, after additional adjustment for multiplicity (by using the Benjamini-Hochberg method), we found that plasma Cer(d18:1/22:0) and Cer(d18:1/24:0) levels maintained conventional statistical significance. In contrast, no significant associations were found between the levels of plasma ceramides and the

severity of coronary stenoses within both RCA and LCX arteries, after adjusting for the above-mentioned established risk factors (data not shown).

As shown in **supplementary Table 1**, almost identical results were found when we excluded from the aforementioned analysis patients with acute ST-elevation myocardial infarction (*n*=15), in which assessment of coronary artery stenosis might be less accurate due to the presence of thrombus within the coronary artery.

Table 4 shows the effect of adjustment for multiple cardiovascular risk factors on the association between each plasma ceramide and the presence of LAD stenosis ≥50% (considered as a dichotomous variable) in the whole sample of patients. Similar to the results above, when the severity of coronary stenosis was included as a continuous measure, higher levels of plasma Cer(d18:1/20:0), Cer(d18:1/22:0) and Cer(d18:1/24:0) were significantly associated with an increased risk of LAD stenosis ≥50%, even after adjustment for multiple cardiovascular risk factors and potential confounding variables (model 2). After further adjustment for multiplicity (by using the Benjamini-Hochberg step-up multiple testing procedure), plasma Cer(d18:1/22:0) and Cer(d18:1/24:0) levels were still independently associated with increased risk of LAD stenosis ≥50%. These results remained unchanged even when patients with acute ST-elevation myocardial infarction (n=15) were excluded from the analysis (**supplementary Table 2**).

Similarly to the results above, when the patients were categorized into three groups according to tertiles of Gensini severity score (which was calculated by summation of the individual coronary segment scores) after excluding patients with acute ST-elevation myocardial infarction, we found that higher levels of plasma Cer(d18:1/20:0), Cer(d18:1/22:0) and Cer(d18:1/24:0) were significantly associated with a greater Gensini

score (included as a categorical variable in multivariable logistic regression models, i.e., 1st tertile *vs*. 2nd tertile and 3rd tertile combined) even after adjustment for multiple cardiovascular risk factors and potential confounding variables (**supplementary Table 3**).

DISCUSSION

The main findings of our study are that higher circulating levels of plasma Cer(d18:1/20:0), Cer(d18:1/22:0) and Cer(d18:1/24:0) were significantly associated with greater angiographic severity of LAD stenosis (included as a continuous measure), as well as with greater risk of LAD stenosis ≥50%, after adjustment for common cardiovascular risk factors, lipid-lowering therapy, e-GFR_{MDRD} and plasma hs-CRP concentrations. Conversely, plasma levels of Cer(d18:1/16:0), Cer(d18:1/18:0) and Cer(d18:1/24:1) were not independently associated with the severity of LAD stenosis in this group of patients. Interestingly, these results were confirmed even when we excluded patients with acute ST-elevation myocardial infarction from the analysis, or when we used the Gensini score for quantifying the angiographic severity of coronary stenoses.

Circulating ceramide levels we observed in our study were well comparable with those measured in other previously published studies including cohorts of patients with CAD or referred for coronary angiography [7,9,10]. At first glance, the lack of a significant association between plasma ceramide levels and the angiographic severity of coronary-artery stenosis within the RCA and LCX arteries might appear unexpected. However, we believe that the most likely explanation for our finding is that the LAD artery was the most commonly occluded coronary artery in these patients (*i.e.,* ~60% of our patients had LAD stenosis ≥50%). In this context, it is also important to underline that the LAD artery and its branches supply most of the inter-ventricular septum and more than half of the left

ventricle and, for this reason, the LAD artery is considered the most important coronary vessel in terms of myocardial blood supply [16]. Therefore, we believe that our finding of a significant positive association between specific plasma ceramide levels and the severity of LAD stenosis might add weight to there being a causal relationship between distinct plasma ceramides and coronary artery stenoses, and further reinforces the view that distinct plasma ceramides (especially those with very long-chain and saturated fat compounds) might play a role in the pathophysiology of CAD (as will be discussed below). However, it should also be considered that we performed coronary angiography, which is an inadequate method for determining key metrics of the coronary vessels, such as diameter of the vessels, extent of disease, and plaque distribution and composition. It is reasonable that the use of intracoronary imaging tools, such as intra-vascular ultrasound and optical coherence tomography, could provide more clear explanation for the significant associations we observed between distinct plasma ceramides and the severity of coronary-artery stenosis within the LAD artery.

An ever-increasing number of observational studies supported a role for distinct plasma ceramides as potential mediators or biomarkers in the development and progression of coronary atherosclerosis [7-11,19]. Currently, it is uncertain whether higher levels of specific ceramides are associated with a greater severity of coronary-artery stenoses in patients with established or suspected CAD. For instance, in the ATHEROREMO-IVUS study, involving 581 patients with stable CAD or suspected ACS, Cheng *et al.* reported that plasma Cer(d18:1/16:0) and Cer(d18:1/24:0) levels were significantly associated with more vulnerable coronary plaque morphology, but not with the severity of coronary stenoses, thus suggesting the existence of a stronger association of specific plasma ceramides with risk of plaque rupture rather than with risk of atherosclerotic plaque progression [13]. In another prospective study of ~500 patients undergoing elective

coronary angiography, Meeusen *et al*. reported that higher plasma Cer(d18:1/16:0), Cer(d18:1/18:0) and Cer(d18:1/24:1) levels were independently associated with a 4-year increased risk of adverse cardiovascular outcomes [10]. However, the authors did not observe any significant association between the aforementioned plasma ceramides and the angiographic severity of coronary-artery stenoses at baseline [10].

The putative underlying mechanisms by which specific plasma ceramides might contribute to the pathophysiology of coronary atherosclerosis are not completely understood. Experimental evidence suggests that different ceramide species are implicated in a variety of key signaling pathways involved in atherogenesis [1,4-6,20,21]. Moreover, it has been shown that the myocardium produces certain ceramides in response to acute ischemia and reperfusion, leading to an increase of specific ceramides that activate mitochondrial autophagy and apoptosis [22,23]. Recently, de Carvalho *et al.* showed myocardial up-regulation of three ceramide-producing enzymes (*i.e*., ceramide synthase 6, serine palmitoyl transferase-2, and neutral sphingomyelinase) in a rodent model of acute myocardial infarction after LAD ligation. In this animal model, there was a net increase in production of long-chain ceramides [mainly Cer(d18:1/20:0), Cer(d18:1/22:0) and Cer(d18:1/24:0)] [24]. Collectively, these experimental findings may help also shed light on the results of our study that showed a strong association between increased levels of these plasma ceramides and severity of LAD stenosis. Moreover, it is also possible to speculate that lipoprotein distribution between the different plasma ceramides might be different, and that might also affect the strength of the associations between distinct plasma ceramides and coronary-artery stenoses. We suggest that further research is needed to better understand the differential role of plasma ceramides with various acyl-chain lengths (and also with different saturated/unsaturated fat compounds) on signaling pathways affecting coronary atherogenesis. Indeed, it is possible to speculate that

differences in the levels of ceramides with various acyl-chain lengths or unsaturated/saturated fat compounds might explain, at least in part, differential risk of developing coronary atherosclerosis [25].

Our study has some important limitations that should be mentioned. Firstly, the cross-sectional design of our study does not allow for establishing the temporality and causality of the observed associations. Secondly, patients were recruited based on their relatively high cardiovascular risk. Hence, our findings might not be generalizable to other cohorts of patients with low or intermediate cardiovascular risk. Thirdly, it was not possible to include a control group of individuals without CAD in this study. Fourthly, although we were able to adjust for several established cardiovascular risk factors, the possibility of residual confounding by unmeasured factors cannot be ruled out. Unfortunately, no information was available on duration of certain diseases/conditions (e.g., smoking, diabetes and dyslipidemia), dietary fat intake, presence of nonalcoholic fatty liver disease (i.e., a pathologic condition associated with increased risk of CAD and where distinct ceramide species have been implicated in the development of hepatic insulin resistance)[26,27], and peri-procedural changes in plasma ceramide levels, in relation to concomitant changes in plasma troponin levels. Fifthly, we did not measure other markers in the ceramide pathway (e.g., sphingomyelins). Finally, after adjusting for multiple comparisons, some of the p-values were of borderline statistical significance [in particular, when we also adjusted for multiplicity using the Benjamini-Hochberg method, we found that plasma Cer(d18:1/22:0) and Cer(d18:1/24:0) levels retained conventional statistical significance]. However, we believe that the consistency of the results across most of the measured plasma ceramides clearly supports the robustness of our observations.

Despite these limitations, our study has also important strengths, such as the relatively large sample size, the consecutive enrolment of patients, the use of coronary angiography for measuring the severity of coronary-artery stenosis, the completeness of database, the adjustment for multiple known cardiovascular risk factors, and the exclusion of patients with a documented history of malignancy, decompensated cirrhosis or kidney failure. In our opinion, the inclusion of patients with such serious co-morbidities might have confounded the interpretation of data.

In conclusion, this cross-sectional study shows for the first time that higher circulating levels of some previously identified high-risk plasma ceramide molecules [mainly plasma Cer(d18:1/20:0), Cer(d18:1/22:0), and Cer(d18:1/24:0)] were significantly associated with a greater angiographic severity of coronary–artery stenosis within the LAD artery among patients who had undergone coronary angiography. These associations were independent of known cardiovascular risk factors, use of lipid-lowering drugs, e-GFR and plasma hs-CRP levels. Our results underscore the fact that measurement of distinct plasma ceramides might be clinically useful in patients with established or suspected CAD. However, further research is needed to better understand whether distinct plasma ceramides may represent new therapeutic targets for the treatment and management of coronary atherosclerosis. Moreover, future studies using intracoronary imaging devices (e.g., intravascular ultrasound or optical coherence tomography) are also needed. Such studies may provide more accurate information about the vessel wall at the site of the coronary stenosis, and provide further insight into the relationship between distinct plasma ceramides and the severity of coronary-artery stenosis within the LAD artery.

Disclosure Statement: All authors declare no conflicts of interest.

Authors' Contributions: AM and GT conceived and designed the study. GL measured plasma ceramides. SB and GC performed coronary angiography and echocardiography. AM, SB, GC, CD, GV and SC researched data and reviewed/edited the manuscript. EB, RL, FB and CDB contributed to discussion and reviewed/edited the manuscript. AM and GT analyzed the data and wrote the manuscript draft. AM and GT are the guarantors of this work and, as such, have full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data. All Authors approved the submitted version of the manuscript.

Funding: GT is supported in part by grants from the University School of Medicine of Verona, Verona, Italy; CDB is supported in part by grants from the Southampton National Institute for Health Research Biomedical Research Centre.

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Table 1. Clinical and biochemical characteristics of 167 consecutive patients with established or suspected CAD undergoing clinically indicated coronary angiography.

	Overall sample
Age (years)	69 ± 10
Sex (men) (%)	73.6
BMI (kg/m^2)	26.3 ± 4
Current smokers (%)	11.4
Systolic blood pressure (mmHg)	142 ± 24
Diastolic blood pressure (mmHg)	72 ± 11
Total cholesterol (mmol/L)	4.51 ± 1.1
LDL-cholesterol (mmol/L)	2.62 ± 0.9
HDL-cholesterol (mmol/L)	1.31 ± 0.3
Triglycerides (mmol/L)	1.40 ± 0.8
Fasting glucose (mmol/L), n=165	6.3 ± 2.1
e-GFR$_{MDRD}$ (mL/min/1.73 m^2)	80.9 ± 21
Troponin I (ug/L), n=164	0.02 (0.01-0.06)
hs-CRP (mg/L)	2.6 (1.1-6.5)
Dyslipidemia (%)	84.4
Hypertension (%)	77.2
Diabetes (%)	21.0
CKD (%)	14.4
CAD (%)	34.1
Valvular heart disease (%)	17.9
Atrial fibrillation (%)	8.9
Left ventricular ejection fraction (%), n=122	56 ± 12
Antiplatelet drug users (%)	53.3
Anticoagulant drug users (%)	14.4
ACE-I/ARB users (%)	60.5
Beta-blocker users (%)	46.1
Diuretic users (%)	33.5
Calcium-channel blocker users (%)	17.9
Nitrate users (%)	14.4
Statin users (%)	44.3
Ezetimibe users (%)	7.2
Fenofibrate users (%)	2.4
Oral hypoglycemic drug users (%), n=35*	17.4
Insulin users (%), n=35*	4.8

Sample size, *n*=167 unless otherwise indicated. Data are expressed as means±SD or medians (interquartile ranges) or percentages.

Abbreviations: ACE-I, angiotensin-converting-enzyme inhibitor; ARB, angiotensin II receptor blocker; BMI, body mass index; CAD, coronary artery disease; CKD, chronic kidney disease; e-GFR$_{MDRD}$, glomerular filtration rate estimated by using the Modification of Diet in Renal Diseases (MDRD) study equation; hs-CRP, high sensitivity C-reactive protein.

Note: Presence of CKD was defined as e-GFR$_{MDRD}$ <60 ml/min/1.73 m^2; dyslipidemia was defined as LDL-cholesterol level ≥2.6 mmol/L (≥100 mg/dL) or use of lipid-lowering drugs; hypertension was defined as blood pressure ≥140/90 mmHg or use of anti-hypertensive drugs. *Information about the use of glucose-lowering drugs were available only for patients with established diabetes (n=35).

Table 2. Coronary angiography data and plasma ceramide levels of 167 consecutive patients with established or suspected CAD undergoing clinically indicated coronary angiography.

	Overall sample
Angiographic coronary data	
Urgent indication, n (%)	30 (18%)
Dominance (right/left/balance), n	154/7/6
Left main stem stenosis (%)	10 (0-20)
LAD stenosis (%)	55 (17-81)
RCA stenosis (%)	40 (0-82)
LCX stenosis (%)	40 (0-76)
Left main stem stenosis ≥50%, n (%)	9 (5.4%)
LAD stenosis ≥50%, n (%)	100 (59.9%)
RCA stenosis ≥50%, n (%)	78 (46.7%)
LCX stenosis ≥50%, n (%)	75 (45.0%)
Stenosis ≥50% in any coronary artery, n (%)	129 (77.2%)
Plasma ceramides	
Cer(d18:1/16:0) (umol/L)	0.329 ± 0.10
Cer(d18:1/18:0) (umol/L)	0.127 ± 0.06
Cer(d18:1/20:0) (umol/L)	0.100 ± 0.04
Cer(d18:1/22:0) (umol/L)	0.725 ± 0.29
Cer(d18:1/24:0) (umol/L)	2.766 ± 1.15
Cer(d18:1/24:1) (umol/L)	1.047 ± 0.37

Sample size, *n*=167. Data are expressed as means±SD or percentages.

Abbreviations: CAD, coronary artery disease; Cer, ceramide; LAD, left anterior descending; LCX, left circumflex; RCA, right coronary artery.

Table 3. Associations between plasma ceramide concentrations and the angiographic severity of coronary stenosis at the level of LAD artery in the whole sample of participants.

Linear Regression Analyses	Standard β coefficient(s)	*P* value
Adjusted model 1		
Cer(d18:1/16:0) (1-SD increment, i.e. 0.10 umol/L)	0.027	0.74
Age (years)	0.100	0.20
Sex (male vs. female)	0.060	0.47
Adjusted model 2		
Cer(d18:1/16:0) (1-SD increment, i.e. 0.10 umol/L)	0.046	0.58
Age (years)	0.051	0.59
Sex (male vs. female)	0.034	0.70
Smoking history (yes vs. no)	0.029	0.82
Prior history of CAD (yes vs. no)	0.157	0.08
Pre-existing diabetes (yes vs. no)	0.021	0.83
Hypertension (yes vs. no)	-0.05	0.59
Dyslipidemia (yes vs. no)	0.076	0.41
hs-CRP (mg/L)	0.083	0.36
e-GFR_{MDRD} (mL/min/1.73 m^2)	-0.03	0.72
Adjusted model 1		
Cer(d18:1/18:0) (1-SD increment, i.e. 0.06 umol/L)	0.106	0.18
Age (years)	0.090	0.25
Sex (male vs. female)	0.071	0.38
Adjusted model 2		
Cer(d18:1/18:0) (1-SD increment, i.e. 0.06 umol/L)	0.134	0.12
Age (years)	0.085	0.34
Sex (male vs. female)	0.042	0.61
Smoking history (yes vs. no)	0.025	0.76
Prior history of CAD (yes vs. no)	0.163	0.06
Pre-existing diabetes (yes vs. no)	0.018	0.83
Hypertension (yes vs. no)	-0.06	0.53
Dyslipidemia (yes vs. no)	0.075	0.37
hs-CRP (mg/L)	0.054	0.53
e-GFR_{MDRD} (mL/min/1.73 m^2)	-0.02	0.83
Adjusted model 1		
Cer(d18:1/20:0) (1-SD increment, i.e. 0.04 umol/L)	0.144	0.06
Age (years)	0.092	0.23
Sex (male vs. female)	0.076	0.33
Adjusted model 2		
Cer(d18:1/20:0) (1-SD increment, i.e. 0.04 umol/L)	0.173	**0.041**
Age (years)	0.086	0.33
Sex (male vs. female)	0.042	0.61
Smoking history (yes vs. no)	0.020	0.80
Prior history of CAD (yes vs. no)	0.175	**0.041**

Pre-existing diabetes (yes vs. no)	0.040	0.64
Hypertension (yes vs. no)	-0.06	0.51
Dyslipidemia (yes vs. no)	0.062	0.46
hs-CRP (mg/L)	0.054	0.53
e-GFR_{MDRD} (mL/min/1.73 m^2)	-0.02	0.86
Adjusted model 1		
Cer(d18:1/22:0) (1-SD increment, i.e. 0.29 umol/L)	0.185	**0.020**
Age (years)	0.138	0.08
Sex (male vs. female)	0.082	0.31
Adjusted model 2		
Cer(d18:1/22:0) (1-SD increment, i.e. 0.29 umol/L)	0.239	**0.005***
Age (years)	0.126	0.15
Sex (male vs. female)	0.040	0.63
Smoking history (yes vs. no)	0.033	0.68
Prior history of CAD (yes vs. no)	0.203	**0.022**
Pre-existing diabetes (yes. vs. no)	0.049	0.56
Hypertension (yes vs. no)	-0.03	0.69
Dyslipidemia (yes vs. no)	0.053	0.53
hs-CRP (mg/L)	0.043	0.61
e-GFR_{MDRD} (mL/min/1.73 m^2)	-0.03	0.75
Adjusted model 1		
Cer(d18:1/24:0) (1-SD increment, i.e. 1.15 umol/L)	0.171	**0.032**
Age (years)	0.141	0.08
Sex (male vs. female)	0.064	0.40
Adjusted model 2		
Cer(d18:1/24:0) (1-SD increment, i.e. 1.15 umol/L)	0.225	**0.009***
Age (years)	0.128	0.15
Sex (male vs. female)	0.022	0.79
Smoking history (yes vs. no)	0.032	0.69
Prior history of CAD (yes vs. no)	0.196	**0.031**
Pre-existing diabetes (yes vs. no)	0.062	0.47
Hypertension (yes vs. no)	-0.03	0.69
Dyslipidemia (yes vs. no)	0.037	0.65
hs-CRP (mg/L)	0.063	0.45
e-GFR_{MDRD} (mL/min/1.73 m^2)	-0.03	0.69
Adjusted model 1		
Cer(d18:1/24:1) (1-SD increment, i.e. 0.37 umol/L)	0.092	0.25
Age (years)	0.086	0.27
Sex (male vs. female)	0.067	0.39
Adjusted model 2		
Cer(d18:1/24:1) (1-SD increment, i.e. 0.37 umol/L)	0.104	0.21
Age (years)	0.076	0.39
Sex (male vs. female)	0.037	0.65

Smoking history (yes vs. no)	0.028	0.72
Prior history of CAD (yes vs no)	0.149	0.09
Pre-existing diabetes (yes. vs no)	0.019	0.81
Hypertension (yes vs. no)	-0.04	0.62
Dyslipidemia (yes vs. no)	0.07	0.39
hs-CRP (mg/L)	0.069	0.42
e-GFR_{MDRD} (mL/min/1.73 m^2)	-0.03	0.74

Sample size, *n*=167. Data are expressed as standardized beta coefficients as tested by linear regression analysis. Severity of LAD stenosis (included as a continuous variable) was the dependent variable in all multivariable linear regression models. Each plasma ceramide was expressed for each standard deviation (SD) increment. For clarity, the significant p-values are highlighted in bold.

NB: Hypertension was defined as blood pressure ≥140/90 mmHg or drug treatment; pre-existing diabetes was defined as self-reported physician-diagnosed diabetes, or use of glucose-lowering medications); dyslipidemia was defined as LDL-cholesterol ≥2.6 mmol/L or drug treatment.

*Adjusted model 2: these associations remained statistically significant even after adjustment for multiplicity by using the Benjamini-Hochberg step-up procedure (with a FDR of 0.05).

Table 4. Associations between plasma ceramide concentrations and presence of LAD stenosis ≥50%, in the whole sample of participants.

Logistic Regression Analyses	Odds Ratio (95% CI)	*P* value
Adjusted model 1		
Cer(d18:1/16:0) (1-SD increment, i.e. 0.10 umol/L)	1.20 (0.86-1.67)	0.27
Age (years)	1.01 (0.86-1.67)	0.39
Sex (male vs. female)	1.63 (0.79-3.38)	0.18
Adjusted model 2		
Cer(d18:1/16:0) (1-SD increment, i.e. 0.10 umol/L)	1.22 (0.86-1.75)	0.27
Age (years)	1.01 (0.98-1.05)	0.41
Sex (male vs. female)	1.53 (0.70-3.35)	0.28
Smoking history (yes vs. no)	1.06 (0.65-1.72)	0.81
Prior history of CAD (yes vs. no)	1.98 (0.91-4.29)	0.08
Pre-existing diabetes (yes vs. no)	1.23 (0.52-2.91)	0.64
Hypertension (yes vs. no)	0.58 (0.24-1.40)	0.22
Dyslipidemia (yes vs. no)	1.37 (0.53-3.53)	0.51
hs-CRP (mg/L)	1.01 (0.99-1.02)	0.34
e-GFR_{MDRD} (mL/min/1.73 m^2)	0.99 (0.98-1.01)	0.45
Adjusted model 1		
Cer(d18:1/18:0) (1-SD increment, i.e. 0.06 umol/L)	1.29 (0.91-1.83)	0.16
Age (years)	1.01 (0.98-1.04)	0.46
Sex (male vs. female)	1.65 (0.80-3.39)	0.17
Adjusted model 2		
Cer(d18:1/18:0) (1-SD increment, i.e. 0.06 umol/L)	1.31 (0.92-1.92)	0.13
Age (years)	1.01 (0.98-1.05)	0.43
Sex (male vs. female)	1.53 (0.69-3.35)	0.29
Smoking history (yes vs. no)	1.07 (0.67-1.74)	0.77
Prior history of CAD (yes vs. no)	2.02 (0.93-4.40)	0.06
Pre-existing diabetes (yes. vs. no)	1.21 (0.51-2.87)	0.66
Hypertension (yes vs. no)	0.54 (0.22-1.31)	0.18
Dyslipidemia (yes vs. no)	1.38 (0.53-3.50)	0.50
hs-CRP (mg/L)	1.01 (0.99-1.02)	0.42
e-GFR_{MDRD} (mL/min/1.73 m^2)	0.99 (0.98-1.01)	0.53
Adjusted model 1		
Cer(d18:1/20:0) (1-SD increment, i.e. 0.04 umol/L)	1.34 (0.96-1.88)	0.08
Age (years)	1.01 (0.98-1.04)	0.44
Sex (male vs. female)	1.66 (0.81-3.39)	0.17
Adjusted model 2		
Cer(d18:1/20:0) (1-SD increment, i.e. 0.04 umol/L)	1.39 (1.00-1.99)	**0.050**
Age (years)	1.01 (0.98-1.05)	0.42
Sex (male vs. female)	1.52 (0.69-3.31)	0.30
Smoking history (yes vs. no)	1.06 (0.65-1.72)	0.82
Prior history of CAD (yes vs. no)	2.12 (0.97-4.64)	0.06
Pre-existing diabetes (yes. vs. no)	1.34 (0.56-3.22)	0.51

Hypertension (yes vs. no)	0.53 (0.21-1.29)	0.16
Dyslipidemia (yes vs. no)	1.29 (0.50-3.36)	0.59
hs-CRP (mg/L)	1.00 (0.99-1.02)	0.40
e-GFR_{MDRD} (mL/min/1.73 m^2)	0.99 (0.98-1.01)	0.55
Adjusted model 1		
Cer(d18:1/22:0) (1-SD increment, i.e. 0.29 umol/L)	1.43 (1.02-2.01)	**0.042**
Age (years)	1.02 (0.99-1.05)	0.21
Sex (male vs. female)	1.66 (0.81-3.41)	0.16
Adjusted model 2		
Cer(d18:1/22:0) (1-SD increment, i.e. 0.29 umol/L)	1.57 (1.08-2.29)	**0.017***
Age (years)	1.02 (0.99-1.06)	0.24
Sex (male vs. female)	1.49 (0.68-3.26)	0.32
Smoking history (yes vs. no)	1.09 (0.67-1.79)	0.72
Prior history of CAD (yes vs. no)	2.38 (1.07-5.32)	**0.029**
Pre-existing diabetes (yes. vs. no)	1.39 (0.58-3.34)	0.46
Hypertension (yes vs. no)	0.57 (0.23-1.41)	0.22
Dyslipidemia (yes vs. no)	1.24 (0.48-3.28)	0.66
hs-CRP (mg/L)	1.00 (0.99-1.02)	0.43
e-GFR_{MDRD} (mL/min/1.73 m^2)	0.99 (0.98-1.01)	0.48
Adjusted model 1		
Cer(d18:1/24:0) (1-SD increment, i.e. 1.15 umol/L)	1.41 (1.00-1.98)	0.05
Age (years)	1.02 (0.99-1.05)	0.19
Sex (male vs. female)	1.56 (0.77-3.17)	0.22
Adjusted model 2		
Cer(d18:1/24:0) (1-SD increment, i.e. 1.15 umol/L)	1.59 (1.08-2.32)	**0.016***
Age (years)	1.02 (0.99-1.06)	0.21
Sex (male vs. female)	1.39 (0.64-3.03)	0.41
Smoking history (yes vs. no)	1.10 (0.68-1.81)	0.69
Prior history of CAD (yes vs. no)	2.38 (1.07-5.33)	**0.029**
Pre-existing diabetes (yes. vs. no)	1.48 (0.61-3.59)	0.38
Hypertension (yes vs. no)	0.56 (0.23-1.39)	0.21
Dyslipidemia (yes vs. no)	1.14 (0.43-3.03)	0.79
hs-CRP (mg/L)	1.00 (0.99-1.02)	0.32
e-GFR_{MDRD} (mL/min/1.73 m^2)	0.99 (0.97-1.01)	0.43
Adjusted model 1		
Cer(d18:1/24:1) (1-SD increment, i.e. 0.37 umol/L)	1.23 (0.88-1.70)	0.22
Age (years)	1.01 (0.98-1.04)	0.52
Sex (male vs. female)	1.59 (0.78-3.24)	0.20
Adjusted model 2		
Cer(d18:1/24:1) (1-SD increment, i.e. 0.37 umol/L)	1.23 (0.87-1.75)	0.24
Age (years)	1.01 (0.98-1.05)	0.49
Sex (male vs. female)	1.47 (0.68-3.20)	0.33
Smoking history (yes vs. no)	1.09 (0.67-1.78)	0.71

Prior history of CAD (yes vs. no)	1.93 (0.89-4.17)	0.08
Pre-existing diabetes (yes. vs. no)	1.23 (0.52-2.90)	0.64
Hypertension (yes vs. no)	0.56 (0.23-1.38)	0.21
Dyslipidemia (yes vs. no)	1.39 (0.54-3.58)	0.49
hs-CRP (mg/L)	1.01 (0.99-1.02)	0.32
e-GFR_{MDRD} (mL/min/1.73 m^2)	0.99 (0.97-1.01)	0.45

Sample size, n=167. Data are expressed as odds ratio and 95% confidence intervals (CI) as tested by univariable and multivariable logistic regression analysis. Presence of LAD stenosis ≥50% (included as categorical variable) was the dependent variable in all multivariable logistic regression models. Each plasma ceramide was expressed for each standard deviation (SD) increment. For clarity, the significant p-values are highlighted in bold.

NB: Hypertension was defined as blood pressure ≥140/90 mmHg or drug treatment; pre-existing diabetes was defined as self-reported physician-diagnosed diabetes, or use of glucose-lowering medications); dyslipidemia was defined as LDL-cholesterol ≥2.6 mmol/L or drug treatment.

*Adjusted model 2: these associations remained statistically significant even after adjustment for multiplicity by using the Benjamini-Hochberg step-up procedure (with a FDR of 0.05).