Association of plasma ceramides with myocardial perfusion in patients with coronary artery disease undergoing stress myocardial perfusion scintigraphy

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ABSTRACT

Objective: It is known that specific plasma ceramides are associated with stress-induced reversible myocardial perfusion defects in patients with established or suspected coronary artery disease (CAD) undergoing myocardial perfusion scintigraphy (MPS). However, it is currently uncertain whether plasma ceramides are also associated with reduced post-stress myocardial perfusion in these patients.

Approach and Results: 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 undergoing stress MPS for clinical indications. Plasma ceramides were measured by a targeted liquid chromatography-tandem mass spectrometry assay both at baseline and after MPS. Multivariable linear regression analysis was undertaken to examine the associations (standardized B coefficients) between plasma ceramides and the percentage of post-stress myocardial perfusion after adjustment for multiple cardiovascular risk factors. Seventy-eight patients had stress-induced myocardial ischemia on MPS (mainly located in the antero-apical wall). Of the six measured plasma ceramides, higher levels of basal Cer(d18:1/18:0) (B=-0.182, p=0.019), Cer(d18:1/20:0) (B=-0.224, p=0.004), Cer(d18:1/22:0) (B=-0.163, p=0.035) and Cer(d18:1/24:1) (B=-0.20, p=0.010) were associated with lower post-stress antero-apical wall perfusion. Notably, these significant associations persisted even after adjustment for conventional cardiovascular risk factors, previous CAD, electrocardiographic left bundle branch block, left ventricular ejection fraction and type of stress testing. Similar results were observed for post-stress plasma ceramides.

Conclusion: Higher circulating levels of specific ceramides, both at baseline and after stress, were independently associated with lower post-stress antero-apical wall perfusion in patients with suspected or established CAD referred for clinically indicated MPS.

LIST OF ABBREVIATIONS

BMI, body mass index
Cer, ceramides
CAD, coronary artery disease
eGFR, estimated glomerular filtration rate
MDRD, Modification of Diet in Renal Disease
MPS, myocardial perfusion scintigraphy
SPECT, single photon emission computed tomography

INTRODUCTION

Ceramides are central molecular species of the sphingolipid pathway, which play a key role as structural lipids and second messengers for intra- and inter-cellular signaling pathways, such as cellular growth, proliferation, differentiation and apoptosis (1,2). Over the last years, the importance of specific plasma ceramides (and their metabolites) as possible mediators or biomarkers of complex disease mechanisms, including atherosclerotic processes, has become increasingly evident (3-5).

Large follow-up studies have recently identified specific plasma ceramides with long and very-long chains [i.e., Cer(d18:1/16:0), Cer(d18:1/18:0) and Cer(d18:1/24:1] as predictors of major cardiovascular events both in patients with established coronary artery disease (CAD) and in those with clinical suspicion for acute coronary syndrome, independent of plasma lipid profile and other traditional cardiovascular risk factors (6-10). In addition, we have recently reported that there was a significant and independent association between these previously identified high-risk plasma ceramides [mainly Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d18:1/22:0) and Cer(d18:1/24:1)] and the presence of inducible myocardial ischemia on myocardial perfusion scintigraphy (MPS), i.e., a reliable, non-invasive test for the diagnosis and prognosis of CAD [10], among patients with established or suspected CAD undergoing stress MPS for various clinical indications (11).

It is important to understand the factors potentially affecting myocardial perfusion after stress MPS in patients with established or suspected CAD, because it is plausible that modifications of these factors may improve myocardial perfusion in situations of stress. The percentage reduction in myocardial perfusion after stress MPS in patients with established or suspected CAD is clinically important, because it reflects the coexistence of coronary atherosclerosis. It is currently not known whether basal and post-stress levels of these plasma ceramides are associated with a reduction in stress-induced myocardial perfusion on MPS. We consider that this topic may be of clinical importance because it is plausible that increases in plasma ceramide levels may worsen myocardial perfusion in patients at high risk of myocardial ischemia.

Therefore, the main aim of this cross-sectional study was to explore whether there was an association between circulating levels of six previously identified high-risk plasma ceramides and post-stress myocardial perfusion in patients with established or suspected CAD undergoing stress MPS.

MATERIALS AND METHODS

Patients

In this analysis, we studied 167 ambulatory patients (128 men and 39 women) with established or suspected CAD, who consecutively underwent stress MPS for various clinical reasons (e.g., chest pain, palpitations, dyspnea, suspected ischemic electrocardiographic alterations, echocardiographic abnormalities or presence of multiple cardiovascular risk factors) at the “IRCCS Sacro Cuore” Hospital of Negrar (Verona) over a 4-month period. We excluded patients with: (*i*) a documented history of malignancy, cirrhosis and kidney failure (defined as estimated glomerular filtration rate <15 ml/min/1.73 m^2 or chronic dialysis); (*ii*) a documented history of overt hyperthyroidism or hypothyroidism; and (*iii*) presence of active infectious diseases (e.g., endocarditis and myocarditis). These 167 patients have been included in a previous study (11).

The local Ethics Committee approved the study protocol. All participants gave their written informed consent for participation in this research.

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 anti-hypertensive drugs. Information on smoking history and 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 Jaffè rate blanked and compensated assay), lipids and other biochemical blood parameters were measured using standard laboratory procedures at the central Laboratory of our hospital. Glomerular filtration rate ($eGFR_{MDRD}$) was estimated using the four-variable Modification of Diet in Renal Disease (MDRD) study equation (12).

Pre-existing CAD was defined as a documented history of myocardial infarction, angina or coronary revascularization procedures. The diagnosis of permanent atrial fibrillation or left bundle branch block 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 heart valve 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 hypoglycemic medications (insulin or oral agents). Dyslipidemia was defined as a total cholesterol level ≥5.2 mmol/L (≥200 mg/dL) and/or drug treatment. Chronic kidney disease was defined as the presence of $eGFR_{MDRD}$ <60 mL/min/1.73 m^2 (12).

Plasma ceramide measurements

Blood samples for ceramide measurements were obtained in ethylenediamine tetra-acetic acid (EDTA)-containing tubes both immediately before and after stress MPS. Plasma was collected within two hours from withdrawal and stored at -80°C until analysis. An expert laboratory physician, who was blinded to clinical details of participants, performed all measurements of plasma ceramides. Ceramides 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) (11,13). Cer(d18:1/17:0) was used as internal standard. The apparatus consisted of an Agilent 1290 UHPLC system coupled with an Agilent 6495 Triple Quadrupole 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 transition was selected to quantify each ceramide. Calibration standards (six points) were daily prepared in surrogate matrix (5% bovine serum albumin) at concentration range 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 coefficient was R^2 >0.99 for all ceramides. Inter-assay and intra-assay coefficients of variations for precision and accuracy for all measured ceramides were less than 15% (11). No matrix interference or carryover was observed.

Myocardial perfusion scintigraphy

Rest and stress studies were performed by an expert nuclear medicine physician, who was blinded to participants' details, in all patients in a two-day (2×370 MBq) protocol using technetium-99m 2-methoxy-isobutyl-isonitrile (99mTc-MIBI), according to local standardized clinical routine (11,14). In total, 94 (56.3%) patients performed an exercise stress testing using bicycle ergometry and 73 (43.7%) patients performed a pharmacological stress testing with dipyridamole infusion. The pharmacological stress testing was used for patients who were unable to perform a standard exercise stress test. During the bicycle exercise test, 99mTc-MIBI was injected at peak symptom-limited exercise testing. Bicycle exercise was performed following the Bruce protocol. Electrocardiogram and blood pressure were recorded at rest and every two minutes during exercise and recovery. 370 MBq of 99mTc-MIBI was injected at rest and at peak exercise. Images were acquired 60 min after the injection of radiopharmaceutical drug on two phases (at baseline and after bicycle ergometry test). The single photon emission computed tomography (SPECT) acquisition protocol usually started with the resting part, followed by SPECT at stress the day after. SPECT was performed using a double-head gamma-camera (Millennium MG, GE) equipped with two scintillation detectors, angled 90°, with high resolution and low energy parallel-hole collimators. The protocol included 32 projections, 40-second projections, and 12 frames per cycle used in association with a 15% window centered on the 140-keV photo peak of 99mTc-MIBI. An expert nuclear medicine physician, who was blinded to the participants' details, including ceramide measurements, classified the amount of inducible myocardial ischemia, according to the number of stress-induced myocardial segments affected (15). Segmental myocardial perfusion was automatically calculated using semi-quantitative analysis of gated myocardial perfusion SPECT images and then the percentages of myocardial perfusion at the level of the antero-apical, lateral-posterior and inferior myocardial walls were calculated from polar maps. Also the ventricular volumes and left ventricular ejection fraction were automatically calculated in all patients, using quantitative analysis of gated myocardial perfusion SPECT images. A similar protocol was used when myocardial perfusion scintigraphy was performed using a pharmacologic stress with intravenous dipyridamole (at a dose of 0.56 mg/kg over 4 minutes) in those patients, who were unable to exercise or who exercised sub-maximally.

Statistical analysis

Data are expressed as means ± SD or percentages. Pearson's correlation coefficients were calculated to examine the univariate linear associations between each plasma ceramide concentration and measures of myocardial perfusion after stress MPS at the level of antero-apical, lateral-posterior and inferior myocardial walls. A multivariable linear regression analysis was used to test the independent association between each plasma ceramide (included as a continuous measure, i.e*.,* for each SD increment) and segmental myocardial perfusion on stress MPS after adjusting 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 dyslipidemia, hypertension, smoking, diabetes, previous CAD, heart rate, electrocardiographic left bundle branch block, left ventricular ejection fraction, and type of stress testing (exercise *vs.* dipyridamole) (model 2). Although this is an exploratory, hypothesis-generating study where an adjustment for multiplicity in multivariable linear regression models is not mandatory, we have also performed a Benjamini-Hochberg method (six plasma ceramides x 1 outcome) that is an adaptative linear step-up multiple testing procedure that control the false discovery rate (FDR) (16). This FDR-controlling procedure provides less stringent control of Type I errors compared to family-wise error rate controlling procedures (such as the Bonferroni correction), which control the

probability of at least one Type I error. Thus, the Benjamini-Hochberg step-up procedure has greater power, at the cost of increased numbers of Type I errors (16). The covariates included in the aforementioned multivariable regression models were selected as potential confounding factors based on their significance in univariate analyses or based on their biological plausibility. A *p*-value <0.05 was considered statistically significant. All statistical analyses were performed using the STATA® software, version 14.2.

RESULTS

Tables 1 and **2** summarize the main clinical/biochemical characteristics, basal and post-stress percentages of myocardial perfusion at the level of antero-apical, lateral-posterior or inferior walls as well as basal and post-stress plasma ceramide concentrations [and their ratios to Cer(d18:1/24:0)] in 167 ambulatory patients with established or suspected CAD, who consecutively underwent stress MPS for various clinical indications. They were predominantly male (77.2%, n=128) and had a mean age of ~70 years; 55.8% had a prior history of CAD, 80% had hypertension, ~95% had dyslipidemia and 24% had established diabetes. Among the different types of statins used by the treated patients (61.8% of total), atorvastatin was the most commonly used (73%), followed by simvastatin (22%) and rosuvastatin (5%). A total of 78 (46.7%) patients had stress-induced reversible myocardial perfusion defects on MPS. Of these patients, 28.8% had one coronary artery affected, 13.2% had two coronary arteries affected and 4.7% had three arteries affected. In particular, these stress-induced reversible myocardial perfusion defects were more commonly observed at the level of the antero-apical wall (75.6% of cases with any myocardial perfusion defect), which is largely supplied by the left anterior descending artery, compared to perfusion defects detected in the lateral-posterior (29.5% of cases with any myocardial perfusion defect) or inferior walls (43% of cases) of the myocardium, which are largely supplied by the circumflex coronary artery and the right coronary artery, respectively.

Supplementary Table 1 summarizes the main clinical/biochemical characteristics, basal and post-stress percentages of myocardial perfusion at the level of antero-apical, lateral-posterior or inferior walls as well as basal and post-stress plasma ceramides in patients stratified by absence or presence of stress-induced reversible myocardial perfusion defects. As expected, compared to those without any myocardial perfusion defect, patients with inducible myocardial ischemia had lower percentages of myocardial perfusion on antero-apical, latero-posterior and inferior wall, both at rest and after stress MPS, even after adjusting for age and sex. Patients with inducible myocardial ischemia also had higher levels of basal Cer(d18:1/20:0), Cer(d18:1/22:0) and Cer(d18:1/24:1) levels compared to those without inducible myocardial ischemia. Conversely, the circulating levels of Cer(d18:1/16:0), Cer(d18:1/18:0) and Cer(d18:1/24:0), both at baseline and after stress, did not significantly differ between the two patient groups.

Supplementary Tables 2-4 show basal and post-stress plasma ceramides in patients stratified by sex, electrocardiographic left bundle branch block or pre-existing diabetes, respectively. Compared to men, women had significantly higher age-adjusted levels of basal and post-stress Cer(d18:1/16:0), Cer(d18:1/18:0) and Cer(d18:1/22:0). Conversely, no significant age- and sex-adjusted differences were found in any measured plasma ceramide levels between patients with and without electrocardiographic left bundle branch block, as well as between patients with and those without pre-existing diabetes, except for post-stress plasma Cer(d18:1/20:0) levels.

Figure 1 shows the scatterplots and correlation coefficients of basal and post-stress plasma Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d18:1/22:0), Cer(d18:1/24:1) [and their specific ratios to Cer(d18:1/24:0), as reported in **Supplementary Figure 1**], with post-stress antero-apical wall perfusion in the whole sample of patients. Specifically, we found that higher levels of these aforementioned ceramides were significantly associated with lower post-stress antero-apical wall perfusion. No significant associations were found between plasma Cer(d18:1/16:0) and Cer(d18:1/24:0) levels and post-stress antero-apical wall perfusion (data not shown). Similarly, there were no significant associations between the levels of these six measured ceramides and post-stress myocardial perfusion at the level of either lateral-posterior or inferior myocardial walls (data not shown).

Table 3 shows the effect of adjustment for multiple cardiovascular risk factors on the associations between basal plasma ceramides and post-stress antero-apical wall perfusion. Higher levels of plasma Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d18:1/22:0) and Cer(d18:1/24:1) were significantly associated with lower post-stress antero-apical wall perfusion after adjustment for age, sex (model 1) or additional adjustment for established cardiovascular risk factors and other potential confounders (model 2). Results remained essentially unchanged even when we further adjusted for systolic blood pressure and treatment for hypertension (instead of “hypertension” included as a single category), or when patients with known diabetes (n=40) were excluded from statistical analyses (data not shown).

Almost identical results were observed when the aforementioned multivariable linear regression analyses were repeated including post-stress plasma ceramides (**Table 4**). Also in such case, post-stress levels of plasma Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d18:1/22:0) and Cer(d18:1/24:1) were inversely associated with post-stress antero-apical wall perfusion after adjusting for several cardiovascular risk factors and potential confounders (models 1 and 2). When we also applied the Benjamini-Hochberg step-up multiple testing procedure in the fully adjusted models 2, we found that basal and post-stress levels of plasma Cer(d18:1/20:0) and plasma Cer(d18:1/22:0) as well as post-stress plasma Cer(d18:1/18:0) levels maintained conventional statistical significance.

DISCUSSION

The novel finding of this exploratory, hypothesis-generating study is that circulating levels of some previously identified high-risk plasma ceramide species are significantly associated with lower post-stress myocardial wall perfusion in patients with history of or clinical suspicion for CAD referred for clinically indicated stress MPS (i.e., an accurate imaging technique for the early diagnosis and quantification of CAD). Specifically, higher circulating levels of Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d18:1/22:0) and Cer(d18:1/24:1), both at baseline and after stress, were associated with lower post-stress antero-apical myocardial wall perfusion, even after adjustment for conventional cardiovascular risk factors, previous CAD, electrocardiographic left bundle branch block, left ventricular ejection fraction and type of MPS stress. In contrast, plasma levels of Cer(d18:1/16:0) and Cer(d18:1/24:0) were not associated with lower antero-apical wall perfusion in this group of high-risk patients.

Overall, these results reinforce and further expand our previous observations showing that higher levels of plasma Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d18:1/22:0) and Cer(d18:1/24:1) were independently associated with the presence of stress-induced reversible myocardial perfusion defects on MPS in this group of patients (11). Notably, these plasma ceramides were found to be significantly associated with stress-induced reversible myocardial perfusion defects also in those patients, who were already treated with a statin (most of whom achieving the target LDL-cholesterol concentrations), and might therefore represent a potential marker of coronary residual risk (11).

At first glance, the lack of any significant association between specific plasma ceramides and post-stress myocardial perfusion at the level of lateral-posterior and inferior walls could appear unexpected. However, the most likely explanation for our finding is that most of the stress-induced reversible myocardial perfusion defects we observed in these patients were mainly located in the anterior-apical wall of the myocardium (~76% of cases). The perfusion of the antero-apical wall is mostly dependent on the left anterior coronary descending artery, which supplies ~45%-55% of the left ventricle and is considered the most critical vessel in terms of myocardial blood supply. Therefore, we consider that the finding of a strong relationship between higher levels of basal and post-stress plasma ceramides and lower post-stress anterior-apical wall perfusion (i.e. the left anterior descending artery area), which was the part of the myocardium more frequently affected by coronary atherosclerosis in our patients with CAD, adds weight to causality and further reinforces the view that specific plasma ceramides may play a role in the pathophysiology of CAD and related clinical outcomes.

In the last few years, plasma ceramides have emerged as potential mediators or biomarkers in many lipid-related diseases, including cardiovascular disease (1,2). Recent longitudinal studies reported that distinct ceramide species are associated with risk of fatal and non-fatal cardiovascular events in high-risk patient populations, independent of coexisting cardiovascular risk factors (6-11,17). For instance, in a recent longitudinal study of 495 patients undergoing elective coronary angiography, Meeusen *et al.* reported that higher levels of plasma Cer(d18:1/16:0), Cer(d18:1/18:0) and Cer(d18:1/24:1) were associated with an increased risk of major adverse cardiovascular events over a follow-up period of 4 years, even after adjustment for age, sex, BMI, hypertension, smoking, lipid profile, plasma glucose levels and family history of CAD (6). In another longitudinal study of 581 patients, who underwent diagnostic coronary angiography or percutaneous coronary intervention for stable angina pectoris or acute coronary syndrome, Anroedh *et al.* showed that higher levels of plasma Cer(d18:1/16:0), Cer(d18:1/20:0) and Cer(d18:1/24:1) were independently associated with adverse cardiovascular outcomes over a median follow-up of nearly 5 years (7). Again, Laaksonen *et al.* combining the data of three prospective cohort studies of patients with stable CAD or acute coronary syndrome found that higher levels of plasma Cer(d18:1/16:0), Cer(d18:1/18:0) and Cer(d18:1/24:1) were associated with cardiovascular mortality, independently of plasma lipid markers and other traditional risk factors (9). Finally, recent analysis of individuals in the PREDIMED (Prevention with Mediterranean Diet) trial, who were at high cardiovascular risk, indicated that higher Cer(d18:1/16:0), Cer(d18:1/22:0), Cer(d18:1/24:0) and Cer(d18:1/24:1) levels were associated with a greater risk of incident cardiovascular events, and that a Mediterranean dietary intervention might mitigate the potential adverse effect of increased plasma ceramide levels on cardiovascular outcomes (8).

To date, the putative pathophysiological mechanisms by which specific plasma ceramides lead to a reduction of myocardial perfusion after stress MPS are not fully understood. Experimentally, it has been shown that different ceramide species play a key role in many atherosclerotic processes, such as lipoprotein aggregation, uptake of lipoproteins and accumulation of cholesterol within macrophages, regulation of nitric oxide synthesis, production of reactive oxygen species, platelet activation, and expression of various cytokines (1,2,18-22). Moreover, experimental studies in animals have shown that ceramides are associated with the development of atherosclerosis (1,2,5). However, further mechanistic studies are needed to better elucidate the role of different plasma ceramides in specific lipid-related diseases, and to examine the differential effects of plasma ceramides with various acyl-chain lengths on multiple signaling processes of coronary atherogenesis. Indeed, it is possible to assume that differences in the levels of ceramides with different acyl-chain lengths might display important alterations in the lipidome, thus contributing to differential risk for atherosclerosis.

Our study has some important limitations that should be mentioned. First, the cross-sectional design of the study does not allow for proving a cause-effect relationship. Second, our sample comprised patients with a relatively high cardiovascular risk. Hence, these results might not be necessarily generalizable to other patient populations with low or intermediate cardiovascular risk. Third, after adjusting for multiple comparisons, some of the *p*-values were of borderline statistical significance (in particular, when we applied the Benjamini-Hochberg step-up multiple testing procedure in our fully adjusted regression models, we found that basal and post-stress levels of plasma Cer(d18:1/20:0) and plasma Cer(d18:1/22:0) as well as post-stress plasma Cer(d18:1/18:0) retained conventional statistical significance). However, we believe that the consistency of the results across most of the measured plasma ceramides supports the robustness of our observations. Finally, we cannot exclude the possibility that other unmeasured confounding factors may partly explain the observed associations.

Despite these limitations, the present study also has important strengths, such as the consecutive enrollment of patients, the relatively large sample size, the use of stress MPS for detecting myocardial perfusion and ischemia, and the exclusion of patients with a documented history of end-stage renal disease, cirrhosis or cancer. We think the inclusion of patients with these comorbid conditions might have confounded the interpretation of data.

In conclusion, this cross-sectional study shows for the first time that specific plasma ceramides, both at baseline and after stress, are significantly associated with lower post-stress antero-apical myocardial perfusion in patients with established or suspected CAD undergoing stress MPS for clinical indications. Although further studies are needed to replicate these findings in larger populations, these results add knowledge in the field; and the data emphasize that measurement of specific plasma ceramides may be warranted in patients with established or suspected CAD. It remains to be proven but we speculate that certain ceramide species could represent new therapeutic targets for the treatment of coronary atherosclerosis.

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Authors' Contributions: AM, SB, MS and GT conceived and designed the study. GL measured plasma ceramide levels. MS performed myocardial perfusion scintigraphy. AM,

SB, GL, CD, MS researched data and reviewed/edited the manuscript. SG, EB, RL 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, had 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.

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HIGHLIGHTS

• Distinct plasma ceramides (CE) are associated with inducible myocardial ischemia in CAD patients.
• It is not known if plasma CE associate with post-stress myocardial perfusion on scintigraphy.
• Distinct plasma CE are associated with post-stress myocardial perfusion on scintigraphy.
• These associations are independent of traditional cardiovascular risk factors.

Table 1. Main clinical and biochemical characteristics in patients with established or suspected CAD undergoing stress MPS.

	Overall sample (n=167)
Age (years)	70.3 ± 9.7
Sex (men) (%)	77.2
Body mass index (kg/m^2)	27.3 ± 4.1
Systolic blood pressure (mmHg)	135 ± 16
Diastolic blood pressure (mmHg)	77 ± 8
Heart rate (bpm)	73 ± 14
Total cholesterol (mmol/L)	4.2 ± 1.0
LDL-cholesterol (mmol/L)	2.5 ± 1.0
HDL-cholesterol (mmol/L)	1.3 ± 0.3
Triglycerides (mmol/L)	1.5 ± 0.7
Fasting glucose (mmol/L), n=82	6.5 ± 2.6
HbA1c (mmol/mol), n=40	48 ± 16
$eGFR_{MDRD}$ (mL/min/1.73 m^2)	79 ± 22
Chronic kidney disease (%)	10.2
Current smokers (%)	8.9
Dyslipidemia (%)	94.6
Hypertension (%)	80.0
Diabetes (%)	24.2
Electrocardiographic left bundle branch block (%)	7.8
Valvular heart disease (%)	7.9
Atrial fibrillation (%)	9.7
Coronary artery disease (%)	55.8
Ischemic stroke (%)	3.6
Left ventricular ejection fraction (%)	55 ± 14
Patients with any perfusion defects on stress MPS (%)	46.7
Number of coronary arteries involved on stress MPS (%)	
zero vessels affected (%)	53.3
1-vessel disease (%)	28.8
2-vessel disease (%)	13.2
3-vessel disease (%)	4.7
Antiplatelet drug users (%)	64.2
Anticoagulant drug users (%)	15.1
Anti-arrhythmic drug users (%)	6.1
Beta-blocker users (%)	47.9
ACE-I/ARB users (%)	61.2
Calcium-channel blocker users (%)	10.9
Diuretic users (%)	28.5
Nitrate users (%)	16.4
Statin users (%)	61.8
Insulin users (%), n=40	3.6
Oral hypoglycemic drug users (%), n=40	18.8

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

Abbreviations: ACE-I, angiotensin-converting-enzyme inhibitor; ARB, angiotensin II receptor blocker; e-GFR_{MDRD}, glomerular filtration rate estimated by using the Modification of Diet in Renal Diseases (MDRD) study equation.

Note: Presence of chronic kidney disease was defined as $eGFR_{MDRD}$ <60 ml/min/1.73 m^2; dyslipidemia was defined as a total cholesterol level ≥5.2 mmol/L or lipid-lowering treatment; hypertension was defined as blood pressure ≥140/90 mmHg or use of any anti-hypertensive agents. HbA1c levels and information regarding the use of glucose-lowering agents were available only for patients with known diabetes mellitus (*n*=40).

Table 2. Basal and post-stress percentages of myocardial wall perfusion and plasma ceramide levels after stress MPS in patients with established or suspected CAD.

	Overall sample (n=167)
Myocardial perfusion during stress MPS	
Antero-apical wall perfusion at rest (%)	71.4 ± 7.7
Lateral-posterior wall perfusion at rest (%)	73.4 ± 13.6
Inferior wall perfusion at rest (%)	62.8 ± 10.6
Antero-apical wall perfusion after stress (%)	73.1 ± 7.9
Lateral-posterior wall perfusion after stress (%)	71.9 ± 9.4
Inferior wall perfusion after stress (%)	62.3 ± 10.7
Plasma ceramides	
Basal Cer(d18:1/16:0) (umol/L)	0.323 ± 0.08
Basal Cer(d18:1/18:0) (umol/L)	0.143 ± 0.07
Basal Cer(d18:1/20:0) (umol/L)	0.106 ± 0.04
Basal Cer(d18:1/22:0) (umol/L)	0.724 ± 0.26
Basal Cer(d18:1/24:0) (umol/L)	2.822 ± 0.78
Basal Cer(d18:1/24:1) (umol/L)	1.158 ± 0.43
Post-stress Cer(d18:1/16:0) (umol/L)	0.328 ± 0.08
Post-stress Cer(d18:1/18:0) (umol/L)	0.148 ± 0.07
Post-stress Cer(d18:1/20:0) (umol/L)	0.107 ± 0.04
Post-stress Cer(d18:1/22:0) (umol/L)	0.730 ± 0.27
Post-stress Cer(d18:1/24:0) (umol/L)	2.858 ± 0.80
Post-stress Cer(d18:1/24:1) (umol/L)	1.197 ± 0.44

Data are expressed as means±SD.

Table 3. Associations between basal plasma ceramides and antero-apical myocardial wall perfusion after stress MPS.

Linear Regression Analyses	Standard β coefficient(s)	*P* value
1-SD increment (+ 0.08 umol/L) in Cer(d18:1/16:0)		
Adjusted model 1	-0.104	0.204
Adjusted model 2	-0.051	0.559
1-SD increment (+ 0.07 umol/L) in Cer(d18:1/18:0)		
Adjusted model 1	-0.200	0.011
Adjusted model 2	-0.161	0.047
1-SD increment (+ 0.04 umol/L) in Cer(d18:1/20:0)		
Adjusted model 1	-0.230	0.003
Adjusted model 2	-0.214	0.008*
1-SD increment (+ 0.26 umol/L) in Cer(d18:1/22:0)		
Adjusted model 1	-0.198	0.010
Adjusted model 2	-0.190	0.012*
1-SD increment (+ 0.78 umol/L) in Cer(d18:1/24:0)		
Adjusted model 1	-0.073	0.349
Adjusted model 2	-0.098	0.220
1-SD increment (+ 0.43 umol/L) in Cer(d18:1/24:1)		
Adjusted model 1	-0.182	0.021
Adjusted model 2	-0.160	0.049

Sample size, *n*=167. Data are expressed as standardized beta coefficients as tested by linear regression analysis. The percentage of antero-apical wall perfusion after stress MPS was the dependent variable. Each ceramide was expressed per 1-SD increment.

Other covariates included in the multivariate linear regression models (along with specific plasma ceramides) were as follows: model 1: adjusted for age and sex; model 2: adjusted for age, sex, diabetes, hypertension (blood pressure ≥140/90 mmHg or drug treatment), dyslipidemia (total cholesterol level ≥5.2 mmol/L or drug treatment), smoking, heart rate, electrocardiographic left bundle branch block, previous CAD, left ventricular ejection fraction, type of stress on MPS (exercise *vs*. dipyridamole).

* Adjusted model 2: these associations remained statistically significant even after adjustment for multiplicity by using the Benjamini-Hochberg step-up procedure.

Table 4. Associations between post-stress plasma ceramides and antero-apical myocardial wall perfusion after stress MPS.

Linear Regression Analyses	Standard β coefficient(s)	*P* value
1-SD increment (+ 0.08 umol/L) in Cer(d18:1/16:0)		
Adjusted model 1	-0.116	0.155
Adjusted model 2	-0.113	0.204
1-SD increment (+ 0.07 umol/L) in Cer(d18:1/18:0)		
Adjusted model 1	-0.204	0.010
Adjusted model 2	-0.189	0.023*
1-SD increment (+ 0.04 umol/L) in Cer(d18:1/20:0)		
Adjusted model 1	-0.223	0.005
Adjusted model 2	-0.234	0.004*
1-SD increment (+ 0.27 umol/L) in Cer(d18:1/22:0)		
Adjusted model 1	-0.181	0.019
Adjusted model 2	-0.195	0.013*
1-SD increment (+ 0.80 umol/L) in Cer(d18:1/24:0)		
Adjusted model 1	-0.067	0.389
Adjusted model 2	-0.120	0.138
1-SD increment (+ 0.44 umol/L) in Cer(d18:1/24:1)		
Adjusted model 1	-0.168	0.034
Adjusted model 2	-0.172	0.039

Sample size, *n*=167. Data are expressed as standardized beta coefficients as tested by linear regression analysis. The mean percentage of antero-apical wall perfusion after stress MPS was the dependent variable. Each ceramide was expressed per 1-SD increment.

Other covariates included in the multivariate linear regression models (along with specific plasma ceramides) were as follows: model 1: adjusted for age and sex; model 2: adjusted for age, sex, diabetes, hypertension (blood pressure ≥140/90 mmHg or drug treatment), dyslipidemia (total cholesterol level ≥5.2 mmol/L or drug treatment), smoking, heart rate, electrocardiographic left bundle branch block, previous CAD, left ventricular ejection fraction, type of stress on MPS (exercise *vs*. dipyridamole).

* Adjusted model 2: these associations remained statistically significant even after adjustment for multiplicity by using the Benjamini-Hochberg step-up procedure.