Plasma concentrations of molecular lipid species predict long-term clinical outcome in coronary artery disease patients

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Running title: Association of molecular lipid species with clinical outcome.

[a]***Abbreviations***

[a] ACS, acute coronary syndrome; CABG, coronary artery bypass grafting; CAD, coronary artery disease; CAG, coronary angiography; CE, cholesteryl ester; Cer, ceramide; IQR, interquartile range; LacCer, lactosylceramide; MACE, major adverse cardiac events; PCI, percutaneous coronary intervention; SAP, stable angina pectoris; STEMI, ST-segment elevation myocardial infarction; UAP, unstable angina pectoris.

Abstract

Purpose: We investigated the associations of ten previously identified high risk molecular lipid species and three ceramide ratios with the occurrence of major adverse cardiac events (MACE) during a median follow-up of 4.7 years in patients with coronary artery disease (CAD).

Methods: Between 2008 and 2011, 581 patients underwent diagnostic coronary angiography or percutaneous coronary intervention for stable angina pectoris (SAP) or acute coronary syndrome (ACS). Blood was drawn prior to the index procedure and lipid species were determined. The primary endpoint was the occurrence of MACE, comprising all-cause mortality, nonfatal ACS or unplanned coronary revascularization. The secondary endpoint comprised all-cause mortality or nonfatal ACS.

Results: During a median follow-up of 4.7 [IQR: 4.2- 5.6] years, 155 patients (27%) had MACE. In multivariable analyses, Cer(d18:1/16:0) concentration was associated with MACE (HR 2.32; 95% CI [1.09-4.96] per ln(pmol/mL) p= 0.030) after adjustment for cardiac risk factors, clinical presentation, statin use at baseline and admission non-HDL cholesterol level. Furthermore, after multivariable adjustment, concentrations of Cer(d18:1/16:0), Cer(d18:1/20:0), Cer(d18:1/24:1) and their ratios to Cer(d18:1/24:0) were associated with the composite endpoint death or nonfatal ACS.

Conclusion: Altogether, the circulating ceramide lipids we investigated here are associated with adverse cardiac outcome during long-term follow-up independent of clinical risk factors.

Keywords

Lipidomics; Ceramides; Heart; Atherosclerosis; Vascular biology; Follow-up; Prognosis.

Introduction

Established lipid markers such as total cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides (TG) and high-density lipoprotein (HDL) cholesterol have long formed the cornerstone of lipid-based risk stratification in coronary artery disease (CAD) (1-4). However these measures alone do not fully capture the complexity of the altered lipid metabolism in cardiovascular disease (2), and this may be the reason that they fail to identify a substantial proportion of patients at high risk for coronary events (1).

Lipidomics is a systems-based study of all lipids (5) that has been defined as the full characterization of lipid molecular species and their biological roles (6). In its most advanced form, lipidomics is able to quantify hundreds of diverse molecular lipid species across multiple lipid classes such as sphingolipids, phospholipids, sterol esters, and acylglycerols, (7) many of which play an integral role in modulation of biological function such as formation of cellular membranes, energy storage and cell signaling (8, 9). Since lipidomics provides such detailed lipid profiles, it may further improve risk stratification of CAD patients and provide novel mechanistic insights into CAD (4).

In line with this hypothesis, we have recently performed lipidomics in the Ludwigshafen Risk and Cardiovascular Health (LURIC) study and identified several molecular lipid species that are associated with fatal events in patients with CAD (1). In the current study, we hypothesized that these ten previously identified high risk molecular lipid species and three ceramide ratios are associated with occurrence of major adverse cardiac events (MACE) during long-term follow-up.

Methods

Study population and design

The design of the European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis (ATHEROREMO) has been described elsewhere in detail (10). In brief, from 2008 until 2011, 581 patients with an indication for diagnostic coronary angiography (CAG) and/or percutaneous coronary intervention (PCI) due to stable angina pectoris (SAP) or acute coronary syndrome (ACS) were included at the Erasmus MC, Rotterdam, the Netherlands. Prior to the CAG or PCI procedure blood samples were collected from the arterial sheath and were transported to the clinical laboratory of Erasmus MC within 2h after blood collection for storage at a temperature of −80 °C. All included patients were 18 years or older. The ATHEROREMO study was approved by the medical ethics committee of Erasmus MC and was performed in accordance with the criteria described in the declaration of Helsinki. Written informed consent was obtained from all included patients.

Serum concentrations of cholesterol and triglycerides

Levels of total cholesterol, LDL cholesterol, HDL cholesterol and TG were measured in the clinical laboratory of the Erasmus MC in serum samples using Roche/Hitachi cobas c 701/702 analyzer (Roche Diagnostics, Indianapolis, USA) on the Cobas 8000 modular analyzer platform (Roche Diagnostics, Indianapolis, USA).

Plasma concentrations of molecular lipids

Molecular lipids and lipid ratios that were previously found to be associated with fatal cardiovascular outcome at a $p < 0.05$ level in the LURIC study were selected for evaluation in the current study(1). These included cholesteryl esters (CE): CE 14:0, CE 18:3, CE 20:4, CE 20:5, CE 22:5; ceramides (Cer): Cer(d18:1/16:0), Cer(d18:1/20:0), Cer(d18:1/24:0), Cer(d18:1/24:1); ceramide ratios Cer(d18:1/16:0)/Cer(d18:1/24:0), Cer(d18:1/20:0)/Cer(d18:1/24:0), Cer(d18:1/24:1)/Cer(d18:1/24:0)), and lactosylceramide (LacCer): LacCer(d18:1/18:0).

Plasma samples for measurement of lipid concentrations were available in 574 patients. Stored plasma samples were subjected to lipid extraction at Zora Biosciences, Finland. Briefly, Samples (10 µL) were spiked with known amounts of lipid-class specific, non-endogenous synthetic internal standards, D6-CE 18:0 (C/D/N Isotopes Inc.,Pointe-Claire, Quebec, Canada), Cer(d18:1/17:0) (Avanti Polar Lipids Inc., Alabaster, AL, USA) and D3-LacCer(d18:1/16:0) (Matreya LLC, State College, Pennsylvania, USA). Lipid extraction was performed using chloroform (HPLC grade) (Rathburn Chemicals Ltd., Walkerburn, Scotland), methanol, and acetic acid (both LC-MS grade) (Sigma-Aldrich GmbH, Steinheim, Germany) (11). After lipid extraction, samples were reconstituted in chloroform: methanol (1:2, v/v) for sphingolipids analysis, and for molecular shotgun lipidomic analysis the extracts were further diluted with chloroform/methanol (1:2, v/v) containing 5 mM ammonium acetate. Quality control samples (QC) were prepared along with the actual samples for lipidomic analyses to monitor the extraction and MS performance. The intra-day (*n=3*) average coefficient of variation (CV) of sphingolipids and CE was less than or equal to 6% and inter-day (*n=24* for Cer and LacCer; n=23 for CE except for CE(22:5) n=22) CV was less than 21% for both sphingolipids and CE.

Sphingolipids were analyzed on a QTRAP® 5500 mass spectrometer (AB SCIEX, Concord, Canada) equipped with an ultra-high pressure liquid chromatography (UHPLC) system CTC PAL autosampler (Leap Technologies) and Accela 1250 Pump (Thermo Fisher Scientific., Massachusetts, United States). Chromatographic separation was performed on an Acquity BEH C18, 2.1 × 50 mm column with a particle size of 1.7 µm (Waters, Milford, MA). Mobile phases were 10 mM ammonium acetate in water with 0.1% formic acid (solvent A) and 10 mM ammonium acetate in acetonitrile:isopropanol (4:3, v/v) containing 0.1% formic acid (solvent B). Lipids were separated with linear gradient from 75% B to 100% B in 15 min. Flow rate was 500 µL/min and column temperature was 60°C. Data was collected using multiple reaction monitoring in positive ion mode (12). Curtain gas was set at 25, ion spray voltage was

set at 5000 and ion source was heated to 400°C. Collision energy was optimized for each lipid class. Collision energy for Cer and LacCer was set to 40 and 45, respectively.

Shotgun lipidomics was performed to monitor CE on a QTRAP® 5500 mass spectrometer (AB SCIEX, Concord, Canada) equipped with a robotic nanoflow ion source NanoMate HD (Advion, NY, USA) as described (11). CE were analyzed in positive ion mode using precursor ion scanning (PIS) of 369.35 with collision energy 30 (13). Mass spectrometry data files were processed using MultiQuant™ 2.0.1 or LipidView™ 1.0 (AB SCIEX, Concord, Canada) (13). Identified lipids were quantified by normalizing against their respective internal standard and volume of plasma used for the extraction. The limit of quantification (LOQ) for Cer, LacCer and CE in extract was 0.0004 µM, 0.0016 µM and 0.012 µM, respectively. All lipids monitored were within the LOQ. The LOQ was defined as the lowest point in the calibration curve with a signal-to-noise ratio greater than or equal to 10.

Follow-up and study endpoints

Clinical and vital status of patients were collected from medical charts, civil registries or by written or telephone contacts with the patients or relatives. All living patients participating in this study received a questionnaire, consisting of queries regarding the occurrence of MACE and re-admissions. For patients with adverse events, hospital discharge letters were obtained and treating physicians or institutions were contacted if necessary for additional information.

The primary endpoint was the occurrence of MACE, comprising all-cause mortality, nonfatal ACS or unplanned coronary revascularization. The secondary endpoint comprised all-cause mortality and nonfatal ACS. ACS was defined as the clinical diagnosis of ST-segment elevation myocardial infarction (STEMI), non-STEMI or unstable angina pectoris (UAP) in accordance with the guidelines of the European society of Cardiology (14, 15). Unplanned coronary revascularization was defined as unplanned repeated PCI or unplanned coronary artery bypass grafting (CABG). The endpoints were adjudicated according to their definitions by a clinical events committee that was blinded to the lipid data.

Statistical analysis

Categorical variables are presented as numbers and percentages. The distributions of continuous variables, including lipid concentrations and lipid ratios, were examined for normality by visual inspection of the histogram. Normally distributed continuous variables are presented as mean± standard deviation (SD). Non-normally distributed continuous variables (which included molecular lipid concentrations and lipid ratios) are presented as median (interquartile range [IQR]) and were logarithmically (Ln) transformed for further analyses.

Patients lost during follow-up were considered at risk until the date of last contact, at which time-point they were censored. Cox proportional hazards models were used to evaluate the associations between molecular lipids and clinical study endpoints. For patients who experienced more than 1 event, the first was considered. The results are presented as hazard ratios (HRs) per unit increase in (Ln-transformed) molecular lipid concentrations or lipid ratios, with 95% confidence intervals (CIs). First, all analyses were performed univariably. In the multivariable analyses, gender, age, hypertension, hypercholesterolemia, diabetes mellitus and statin use were considered as potential confounders and were entered as covariates. These covariates were chosen for etiologic reasons and were based on existing literature (16). To evaluate whether the associations between molecular lipids and the clinical endpoints are independent of serum LDL cholesterol levels or serum non-HDL cholesterol levels, baseline serum LDL cholesterol level and baseline serum non-HDL cholesterol level were additionally (and consecutively) added into the multivariable models. Serum non-HDL level was calculated by subtracting HDL cholesterol level from total cholesterol level. In the full cohort, indication for CAG (ACS versus SAP) was also entered as a covariate. Interaction terms were added to the model to account for possible effect modification by indication for baseline CAG. Subsequently, analyses were stratified on indication for CAG. All data were analyzed with SPSS software (SPSS 23.0 IBM corp., Armonk, NY, USA). All statistical tests were two-tailed and p-values <0.05 were considered statistically significant.

Results

Baseline characteristics

The baseline clinical characteristics and the lipid concentrations of the ATHEROREMO study are summarized in Table 1 and Table 2. In total 574 patients were included. The mean age of the patients was 61.5 years and 75% were men. A total of 55% patients were diagnosed with ACS (28% STEMI and 26% non-STEMI), and 46% patients with SAP. PCI was performed in 88% of the patients during the index procedure. Prior to the index procedure median serum LDL cholesterol level was 2.71 [IQR: 2.12- 3.54] mmol/l, median serum HDL cholesterol level was 1.04 [IQR: 0.87- 1.29] mmol/l, median serum non-HDL cholesterol level was 3.23 [2.54- 4.00] mmol/l, and median serum triglyceride (TG) level was 1.27 [IQR: 0.88- 1.83] mmol/l in the full cohort. ACS patients had significantly higher serum LDL cholesterol level ((median: 3.10 [IQR: 2.32- 3.87] mmol/l) p= < 0.001), higher serum non-HDL cholesterol level ((median: 3.56 [2.81- 4.36]mmol/l) p = < 0.001) and lower serum TG level ((median= 1.15 [IQR: 0.77 - 1.77] mmol/l) p = < 0.001)) compared with SAP patients (median: 2.37 [IQR: 1.94- 2.99] mmol/l, 2.83 [2.35- 3.56] mmol/l and 1.41 [IQR: 1.05- 1.94] mmol/l, respectively). In addition, several other clinical characteristics were significantly different between the ACS patients and the SAP patients (Table 1). At the time of hospital admission 89% of the patients in the full cohort used statins.

In Table 2 ACS patients had significantly higher plasma concentrations of CE 22:5; Cer(d18:1/16:0), Cer(d18:1/20:0), Cer(d18:1/24:0), Cer(d18:1/24:1), LacCer(d18:1/18:0) and Cer(d18:1/16:0)/Cer(d18:1/24:0) in the full cohort and in patients with no event (the non-MACE cohort) as compared with SAP patients. In patients with an event (MACE cohort), except plasma concentration of LacCer(d18:1/18:0), all of the above mentioned lipid species plasma concentrations were significantly higher in the ACS patients as compared with SAP patients. In addition, in ACS patients concentration of Cer(d18:1/16:0) was significant higher (p=0.054) in the MACE cohort as compared with the non-MACE cohort.

Molecular lipids concentrations and cardiovascular outcome

In the full cohort (n=574) vital status was acquired for 572 patients (99.7%). The follow-up questionnaire assessing the occurrence of MACE was completed by 99% of the 574 patients. During a median follow-up time of 4.7 years (IQR: [4.2- 5.6]) years, a total of 155 patients (27%) experienced at least 1 MACE (primary endpoint). In the ACS group, 65 patients (21%) experienced MACE during long-term follow-up, in the SAP group this was 90 patients (34%).

The results for the associations between the molecular lipids concentrations and MACE are depicted in Figure 1 and Supplemental Table S1a. In multivariable analyses, after adjustment for cardiac risk factors, clinical presentation and statin use at baseline, Cer(d18:1/16:0) concentration (HR: 2.14; 95% CI [1.22- 3.76] per ln(pmol/mL) p= 0.008) and Cer(d18:1/24:1) concentration (HR: 1.64; 95% CI [1.00-2.68] per ln(pmol/mL) p= 0.049) were significantly associated with MACE. After additional adjustment for admission serum LDL cholesterol level, or serum non-HDL cholesterol level, only Cer(d18:1/16:0) concentration remained associated with MACE (HR: 2.16; 95% CI [1.09- 4.27] per ln(pmol/mL), p= 0.027, and HR: 2.32; 95% CI [1.09- 4.96] per ln(pmol/mL, p= 0.030, respectively).

The interaction term between Cer(d18:1/16:0) and indication for CAG was significant (p= 0.030) in the multivariable model. In ACS patients, higher Cer(d18:1/16:0) concentration was significantly associated with MACE, both in the uni- and multivariable model (HR adjusted for cardiac risk factors, statin use and non-HDL cholesterol level at baseline: 6.13; 95% CI [1.65 - 22.8]) per ln(pmol/mL) p= 0.007) (Supplemental Table S2a). In SAP patients, HRs were closer to the null and did not reach statistical significance (Supplemental Table S2b). The interaction term between Cer(d18:1/16:0)/Cer(d18:1/24:0) and indication for CAG was also significant (p= 0.044). Its association with MACE only reached statistical significance, in univariable analysis in ACS patients (Supplemental table S2a).

The incidence of MACE for Cer(d18:1/16:0) levels above and below the median are depicted in Figure 3. After adjustment for cardiac risk factors, statin use and baseline serum non-HDL cholesterol level, plasma Cer(d18:1/16:0) levels above vs below the median were significantly associated with

MACE (HR: 2.63; 95% CI [1.44- 4.83], per ln(pmol/mL) p= 0.002) in ACS patients. In the full cohort and in SAP patients significant associations between plasma Cer(d18:1/16:0) levels above vs below the median and MACE could not be demonstrated (Figure 3).

Several lipid species displayed associations with the secondary endpoint (Figure 2 and Supplemental Table S1b); in univariable analysis, concentrations of Cer(d18:1/16:0), Cer(d18:1/20:0), Cer(d18:1/24:1), LacCer(d18:1/18:0), Cer(d18:1/16:0)/Cer(d18:1/24:0), Cer(d18:1/20:0)/Cer(d18:1/24:0) and Cer(d18:1/24:1)/Cer(d18:1/24:0) were significantly associated with the composite endpoint of death or nonfatal ACS (Supplemental table S1b). After multivariable adjustment for cardiac risk factors, indication for CAG, statin use at baseline and serum LDL cholesterol level, except LacCer(d18:1/18:0) concentration, all of the above mentioned lipid species remained significantly associated with the composite endpoint of death or nonfatal ACS. Results remained materially the same after adjusting the multivariable models for serum non-HDL cholesterol level instead of serum LDL cholesterol level. The interaction terms between indication for CAG and Cer(d18:1/16:0) (p= 0.006), Cer(d18:1/24:1) (p= 0.025), Cer(d18:1/16:0)/Cer(d18:1/24:0) (p= 0.004) and Cer(d18:1/24:1)/Cer(d18:1/24:0) (p= 0.006) were significant on univariable and multivariable adjustment. All these associations were driven by the ACS patients (Supplemental table S3a and S3b). Interaction terms between indication for CAG and the remaining molecular lipids and lipid ratios did not reach statistical significance.

Discussion

We investigated the associations of ten previously identified high risk molecular lipid species and three ceramide ratios with clinical cardiovascular outcome during long-term follow-up in 581 patients. The main finding of our study was that higher Cer(d18:1/16:0) concentration and Cer(d18:1/24:1) concentration were significantly associated with MACE after multivariable adjustment for cardiac risk factors, clinical presentation and statin use at baseline. After additional adjustment for admission serum LDL cholesterol level or non-HDL cholesterol level, this association persisted only for Cer(d18:1/16:0) concentration. The latter association was driven by patients that presented with ACS. Another important

finding of this study was that several lipid species were significantly and independently associated with the secondary endpoint, comprising the composite of all-cause mortality or nonfatal ACS. Likewise, several of these associations were driven by patients presenting with ACS, in whom we also observed higher plasma lipid concentrations as compared to patients with SAP.

Ceramides are a family of waxy lipid molecules and are composed of sphingosine and a fatty acid (17). They can be implicated in coronary artery disease through several mechanisms(3, 8). Ceramide is mainly produced through the SMase pathway, which breaks down sphingomyelin in the cell membrane and releases ceramides (17, 18). The production of ceramides can be increased by numerous cardiovascular risk factors such as oxidized-LDL and homocyteine (17). Moreover, inflammatory cytokines could also activate SMase and increase ceramide production mediated by increased reactive oxygen species (ROS) (17), which include H_2O_2, superoxide and hydroxyl radicals(17, 19). Ceramides can also act as signaling molecules regulating numerous cell responses and functions including the differentiation, proliferation, apoptosis and gene expression such as cytokines(17). Some of these roles of ceramides are associated with the molecular mechanisms of atherosclerosis and with plaque vulnerability (1, 17, 20).

The molecular lipids species in this study were chosen from the LURIC lipidomic study (1). The LURIC lipidomic study compared 258 male CAD patients who died within 3 years of follow-up with 187 matched control patients with CAD who did not die during the follow up. The chosen molecular lipid species were associated with CAD outcome at the $p<0.05$ level. Our present results in essence validate the previous LURIC study, albeit with a somewhat different endpoint that also contains non-fatal adverse cardiovascular events. Several molecular lipid species such as CE 14:0, CE 18:3, CE 20:4, CE 20:5, CE 22:5, Cer(d18:1/24:0) were protective in the LURIC study but were not associated with clinical outcome in the present study. Conversely, Cer(d18:1/16:0), Cer(d18:1/20:0) and Cer(d18:1/24:1) were associated with mortality in the LURIC study and with clinical outcome in our present study. In a previous study ceramide long-chain-species were shown to mediate insulin resistance in mice and to be pro-apoptotic, whereas very-long-chain species were anti-apoptotic (21, 22). In our present study Cer(d18:1/16:0) and

Cer(d18:1/20:0) were independently associated with clinical outcome and more harmful than Cer(d18:1/24:0). Interestingly Cer(d18:1/24:1) behaved contrarily when compared with Cer(d18:1/24:0). The reason for this difference remains to be investigated in further studies. Furthermore, in the LURIC study LacCer(d18:1/18:0) was associated with mortality, whereas in the current study there was only an association with clinical outcome (Supplemental Table S1B) in the unadjusted model. Based on previous observations LacCer (a glycosphingolipid) and other glucosylceramides appear to influence the atherogenic process in the atherosclerotic plaque by suppressing the production of macrophage apolipoprotein E leading to an accumulation of cholesterol in macrophage foam cells (19, 23). Moreover, in aortic smooth muscle cell LacCer is activated by oxidized-LDL and subsequently, LacCer enhances the activity of nicotinamide adenine dinucleotide phosphate oxidase to generate superoxide radicals which in turn mediate p44MAPK activation to enhance nuclear transcription factor expression and to stimulate the proliferation of smooth muscle cells, thereby contributing to atherosclerosis(2, 23). Altogether, as increased levels of LacCer mediate plaque formation, a relationship between LacCer and cardiovascular clinical outcome is to be expected. However in our study there was no independent association. Further studies are needed to establish the biological mechanisms of LacCer in CAD patients.

In our previous report on the current study population, Cer(d18:1/16:0) was an independent predictor of MACE during 1 year follow-up, and the three ceramide ratios were independent predictors of the composite endpoint of death or nonfatal ACS (20). Our current study confirms and extends the findings of our previous lipidomics analyses with shorter term follow-up (20).

Associations of lipids with incident acute MACE (all-cause mortality and nonfatal ACS) were more prominent than those with 'overall' MACE. Associations were also more prominent in patients presenting with ACS than in those with SAP; although in the stratified analyses numbers of MACE were limited (90 in SAP patients and 65 in ACS patients), which may have influenced statistical significance, hazard ratios in SAP patients were also clearly closer to the null and interaction terms were significant. These findings are in line with pathophysiological insights from earlier studies. Patients with ACS have been shown to exhibit an increased pro-inflammatory and oxidative state compared to SAP patients (24,

25). This state even persists after stabilization (26, 27). High lipid plasma concentrations induce oxidative stress mediated by ROS (24, 28) and herewith further increase the risk of incident ACS. Furthermore, in ACS patients, so-called high-risk or vulnerable plaques have been shown to be more frequently present, which are more likely to lead to plaque rupture and thus to acute cardiac events (29, 30). These plaques have been shown to carry large lipid cores, and have been associated with high circulating lipid levels (20, 31).

In the last decade lipid species have received considerable attention as potential biomarkers in several lipid-related diseases (9). Furthermore, several molecular lipid species have been associated with the composition of atherosclerotic plaque (20) and with cardiovascular events during short-term follow-up (20, 32). However, clinical studies in patients with CAD on the association of lipid molecular species with cardiovascular outcome during long term follow-up are scarce. Laaksonen et al. (32) examined several ceramides and ceramide ratios in the Corogene cohort (80 stable CAD patients who died and 80 matched controls, 2.5 years follow-up), as well as in the BECAC cohort (1580 stable CAD patients, 81 of whom died during 4.6 years follow-up). In both cohorts Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:1), Cer(d18:1/16:0)/Cer(d18:1/24:0), Cer(d18:1/18:0)/Cer(d18:1/24:0) and Cer(d18:1/24:1)/Cer(d18:1/24:0) were associated with cardiovascular death. Although Laaksonen et al. only examined cardiovascular death, and not (acute) MACE, the results with regard to the corresponding ceramides and ceramide ratios were in line with our results, even though the associations in our study were driven by patients presenting with ACS. Havulinna et al. (3), in the FINRISK cohort, measured 4 circulating ceramides in a healthy population, and within this population they examined a subgroup with prevalent or incident MACE. In these 396 patients, plasma Cer(d18:1/16:0) and Cer(d18:1)/24:1) concentrations were independent predictors of recurrent MACE (n=226) or fatal recurrent MACE (n=70) during a follow-up of 13 years. These results were in line with ours, although in our study the association between Cer(d18:1/24:1) and MACE was not independent of LDL or non-HDL cholesterol level. Conversely, its association with the secondary endpoint (nonfatal ACS or death) was independent of LDL or non-HDL cholesterol level. Other studies on long-term prognostic value of lipidomics have mostly

used healthy populations. In the Bruneck study, Stegemann et al.(33) analyzed the association of 135 lipid species (including CEs) with incident cardiovascular disease (CVD) during 10-year follow-up in a prospective population-based survey. They demonstrated significant associations for 28 lipids. Among these lipid species, 3 were most informative for CVD risk: TAG(54:2), CE(16:1) and PE(36:5). The results with regard to the CEs that were also measured in this study were in line with our results, i.e. no significant associations were found. Moreover, Alshehry et al. (2) examined the prognostic value of 310 plasma lipid species (including CEs and CERs) for cardiovascular risk stratification in a case-cohort of 3779 individuals with diabetes mellitus that included 698 patients with cardiovascular events and 355 patients with cardiovascular death. Multiple lipid species were significantly associated with cardiovascular events and cardiovascular death. In line with our study, a significant association was found between Cer(d18:1/24:1) and death. In contrast to our study, no association was found between plasma Cer(d18:1/16:0) and cardiovascular outcome. This may possibly be explained by differences in the study populations; in particular, the patients studied by Alshehry et al. were diagnosed with diabetes mellitus and had ≥ 1 additional cardiovascular risk factors. For the other lipid species, no significant associations were found.

Some limitations of this study need to be acknowledged. First, this study is an observational cohort study. Despite using multivariable analysis to adjust for possible confounders that may be related to the study outcomes, we cannot exclude the possibility of residual confounding. Secondly, a large proportion of the subjects were on lipid lowering medication, which will influence the plasma lipid concentrations in these individuals. However, all multivariable analyses were adjusted for statin use.

In conclusion, in patients with established CAD, plasma Cer(d18:1/16:0) was associated with MACE during a median follow-up time of 4.7 years, independently from established cardiac factors, statin use and serum LDL or non-HDL cholesterol level. Furthermore, after multivariable adjustment, concentrations of Cer(d18:1/16:0), Cer(d18:1/20:0), Cer(d18:1/24:1) and all ceramide ratios were associated with the secondary endpoint, comprising the composite of all-cause mortality or nonfatal ACS. Our results support the hypothesis that ceramide plasma concentrations and ratios predict long-term

cardiovascular outcome and therefore circulating molecular lipids may further improve risk stratification of CAD patients.

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Disclosures

Mika Hilvo, Dimple Kauhanen, Kaisa Koistinen, and Reijo Laaksonen are employed by Zora Biosciences, Espoo, Finland. Other authors declare no conflict of interest.

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Tables

Table 1. Clinical characteristics

Clinical characteristics	Total (n=574)	ACS patients (n=313)	SAP patients (n= 261)	p-value
Age, years, mean ± SD	61.5 ± 11.3	59.7 ± 11.9	63.6 ± 10.3	<0.001
Male, n (%)	432 (75)	230 (74)	202 (77)	0.279
Diabetes Mellitus, n (%)	97 (17)	40 (13)	57 (22)	0.004
Hypertension, n (%)	298 (52)	137 (44)	161 (62)	<0.001
Hypercholesterolemia, n (%)	318 (55)	138 (44)	180 (69)	<0.001
Smoking, n (%)	166 (29)	116 (37)	50 (19)	<0.001
Positive family history of CAD, n (%)	298 (52)	145 (46)	153 (59)	0.004
Previous MI, n (%)	184 (32)	80 (26)	104 (40)	<0.001
Previous PCI, n (%)	184 (32)	57 (18)	127 (49)	<0.001
Previous CABG, n (%)	18 (3)	7 (2)	11 (4)	0.176
Previous stroke, n (%)	26 (5)	11 (4)	15 (6)	0.200
Peripheral artery disease, n (%)	35 (6)	11 (4)	24 (9)	0.005
History of heart failure, n (%)	19 (3)	6 (2)	13 (5)	0.041
Serum LDL cholesterol, mmol/L	2.71 [2.12-3.54]	3.10 [2.32-3.87]	2.37 [1.94-2.99]	<0.001
Serum HDL cholesterol, mmol/L	1.04 [0.87-1.29]	1.05 [0.87-1.27]	1.03 [0.86-1.30]	0.80
Serum non-HDL levels, mmol/L	3.23 [2.54-4.00]	3.56 [2.81-4.36]	2.83 [2.35-3.56]	<0.001
Serum TG mmol/L	1.27 [0.88-1.83]	1.15 [0.77-1.77]	1.41 [1.05-1.94]	<0.001
Statin use at baseline, n (%)	508 (89%)	308 (98%)	235 (90%)	0.499
Procedural characteristics				
Indication for CAG				
ACS, n (%)	313 (55)	313 (100)	0 (0)	
STEMI, n (%)	162 (28)	162 (52)	0 (0)	
Non-ST-elevation, n (%)	151 (26)	151 (48)	0 (0)	
Stable angina pectoris, n (%)	261 (46)	0 (0)	261 (100)	
PCI performed, n (%)	505 (88)	291 (93)	214 (82)	
Coronary artery disease *				
No significant stenosis, n (%)	42 (7)	18 (6)	24 (9)	
1-vessel disease, n (%)	304 (53)	172 (55)	132 (51)	
2-vessel disease, n (%)	167 (29)	88 (28)	79 (30)	
3-vessel disease, n (%)	61 (11)	35 (11)	26 (10)	

* A significant stenosis was defined as a stenosis ≥ 50% of the vessel diameter by visual assessment of the coronary angiogram.

Continuous variables are presented as mean± standard deviation (SD) or median [IQR]. Categorical variables are presented in numbers (n) and percentages (%).

P-value was obtained from student's t-test.

ACS, acute coronary syndrome; CABG, coronary artery bypass grafting; CAD, coronary artery disease; CAG, coronary angiography; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; MI, myocardial infarction; PCI, percutaneous coronary intervention; SAP, stable angina pectoris; TG, triglycerides.

Table 2. Lipid concentrations in the full cohort, MACE cohort and non-MACE cohort

Lipid concentrations in the full cohort	Total (n=574)	ACS patients (n=313)	SAP patients (n= 261)	p-value
CE 14:0, pmol/µl	21.7 [15.9-28.1]	22.9 [16.5-30.5]	21.2 [15.4-26.8]	0.008
CE 18:3, pmol/µl	70.3 [51.8-90.7]	72.3 [53.6-99.5]	66.1 [50.3-85.2]	0.003
CE 20:4, pmol/µl	386 [317-457]	394 [324-453]	374 [307-471]	0.31
CE 20:5, pmol/µl	49.1 [36.3-72.6]	49.2 [36.4-72.1]	49.0 [35.9-74.7]	0.69
CE 22:5, pmol/µl	2.65 [2.00-3.62]	2.81 [2.12-3.77]	2.53 [1.90-3.40]	0.037
Cer(d18:1/16:0) pmol/µl	0.12 [0.10-0.15]	0.13 [0.11-0.17]	0.11 [0.09-0.13]	<0.001
Cer(d18:1/20:0) pmol/µl	0.11 [0.09-0.15]	0.12 [0.10-0.16]	0.11 [0.08-0.13]	<0.001
Cer(d18:1/24:0) pmol/µl	5.98 [4.72-7.49]	6.43 [5.00-8.07]	5.65 [4.49-6.61]	<0.001
Cer(d18:1/24:1) pmol/µl	1.79 [1.42-2.25]	1.89 [1.52-2.44]	1.67 [1.35-2.05]	<0.001
LacCer(d18:1/18:0) pmol/µl	0.13 [0.10-0.16]	0.13 [0.11-0.16]	0.12 [0.10-0.15]	0.001
Cer(d18:1/16:0)/Cer(d18:1/24:0) pmol/µl	0.020 [0.018-0.024]	0.021 [0.018-0.025]	0.020 [0.017-0.023]	0.001
Cer(d18:1/20:0)/Cer(d18:1/24:0) pmol/µl	0.019 [0.016-0.024]	0.019 [0.015-0.024]	0.019 [0.016-0.023]	0.62
Cer(d18:1/24:1)/Cer(d18:1/24:0) pmol/µl	0.31 [0.26-0.36]	0.31 [0.26-0.36]	0.31 [0.26-0.36]	0.65
Lipid concentrations in the MACE cohort*	**Total (n=155)**	**ACS patients (n=65)**	**SAP patients (n=90)**	**P-value**
CE 14:0, pmol/µl	22.6 [15.7-27.1]	21.7 [15.5-30.3]	22.7 [15.7-26.6]	0.67
CE 18:3, pmol/µl	67.9 [51.2-90.3]	70.3 [52.8-103]	66.9 [50 -84.1]	0.17
CE 20:4, pmol/µl	381 [310-445]	381 [310-432]	379 [310-447]	0.95
CE 20:5, pmol/µl	52.4 [37.4-74.5]	52.6 [38.4-76.3]	50.9 [36.6-74.4]	0.73
CE 22:5, pmol/µl	2.57 [1.86-3.71]	2.61 [2.04-3.97]	2.51 [1.80-3.45]	0.065
Cer(d18:1/16:0) pmol/µl	0.12 [0.10-0.16]	0.15 [0.11-0.17]	0.11 [0.09-0.13]	<0.001
Cer(d18:1/20:0) pmol/µl	0.11 [0.09-0.16]	0.13 [0.10-0.17]	0.11 [0.08-0.14]	0.011
Cer(d18:1/24:0) pmol/µl	5.86 [4.65-7.48]	6.44 [5.02-8.10]	5.66 [4.54-6.58]	0.063
Cer(d18:1/24:1) pmol/µl	1.78 [1.35-2.36]	2.10 [1.66-2.84]	1.62 [1.33-2.13]	0.008
LacCer(d18:1/18:0) pmol/µl	0.13 [0.10-0.16]	0.14 [0.10-0.18]	0.13 [0.10-0.16]	0.137
Cer(d18:1/16:0)/Cer(d18:1/24:0) pmol/µl	0.021 [0.018-0.025]	0.022 [0.019-0.027]	0.019 [0.017-0.024]	0.006
Cer(d18:1/20:0)/Cer(d18:1/24:0) pmol/µl	0.020 [0.016-0.025]	0.020 [0.016-0.024]	0.020 [0.015-0.025]	0.504
Cer(d18:1/24:1)/Cer(d18:1/24:0) pmol/µl	0.31 [0.27-0.37]	0.33 [0.27-0.36]	0.31 [0.26-0.37]	0.222
Lipid concentrations in the non-MACE cohort**	**Total (n=411)**	**ACS patients (n=242)**	**SAP patients (n=169)**	P-value
CE 14:0, pmol/µl	21.5 [15.8-28.7]	23 [16.5-30.5]	20.7 [15.3-27]	0.007
CE 18:3, pmol/µl	70.5 [51.7-91.3]	73.8 [53.5-99]	66 [50.3 -86.4]	0.011
CE 20:4, pmol/µl	391 [321-467]	397 [310-432]	374 [304-476]	0.272
CE 20:5, pmol/µl	49.1 [35.6-70.5]	49.2 [35.5-70.7]	47.5 [35.6-70]	0.575
CE 22:5, pmol/µl	2.69 [2.04-3.58]	2.79 [2.12-3.70]	2.54 [1.92-3.40]	0.041
Cer(d18:1/16:0) pmol/µl	0.12 [0.10-0.15]	0.13 [0.11-0.16]	0.11 [0.09-0.13]	<0.001
Cer(d18:1/20:0) pmol/µl	0.12 [0.09-0.14]	0.12 [0.09-0.15]	0.11 [0.08-0.13]	<0.001
Cer(d18:1/24:0) pmol/µl	6 [4.75-7.47]	6.39 [4.97-8.07]	5.64 [4.49-6.64]	<0.001
Cer(d18:1/24:1) pmol/µl	1.78 [1.35-2.36]	1.86 [1.51-2.33]	1.68 [1.35-2.04]	<0.001
LacCer(d18:1/18:0) pmol/µl	0.13 [0.10-0.16]	0.13 [0.11-0.16]	0.12 [0.10-0.15]	0.001
Cer(d18:1/16:0)/Cer(d18:1/24:0) pmol/µl	0.021 [0.018-0.025]	0.021 [0.018-0.024]	0.020 [0.017-0.023]	0.017
Cer(d18:1/20:0)/Cer(d18:1/24:0) pmol/µl	0.020 [0.016-0.025]	0.019 [0.015-0.024]	0.019 [0.016-0.023]	0.60
Cer(d18:1/24:1)/Cer(d18:1/24:0) pmol/µl	0.31 [0.27-0.37]	0.30 [0.25-0.36]	0.31 [0.26-0.36]	0.77

* Patients with a major adverse cardiac event

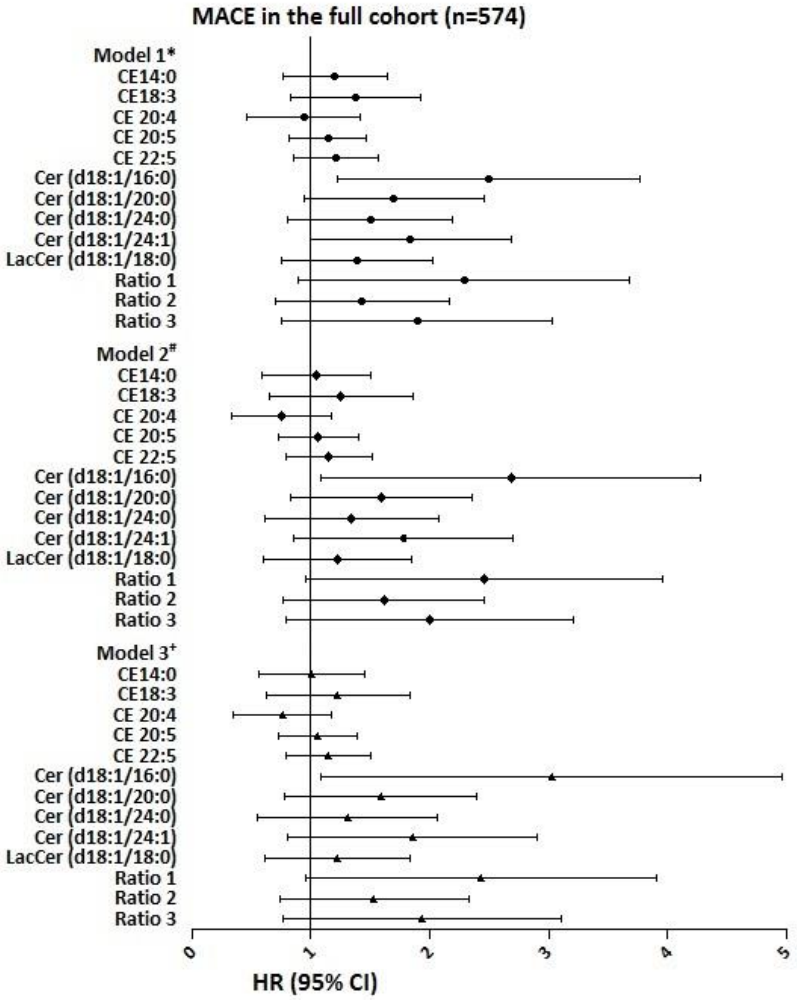
** Patients with no major adverse cardiac event

Concentrations are presented in µM as median [IQR].

P-value was obtained from student's t-test for difference in ln-transformed mean lipid concentration.

ACS, acute coronary syndrome; CE, cholesteryl ester; Cer, ceramide; LacCer, lactosylceramide; MACE, major adverse cardiac events SAP, stable angina pectoris.

Figures



* Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus and statin use.

Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus, statin use and serum LDL cholesterol level at baseline.

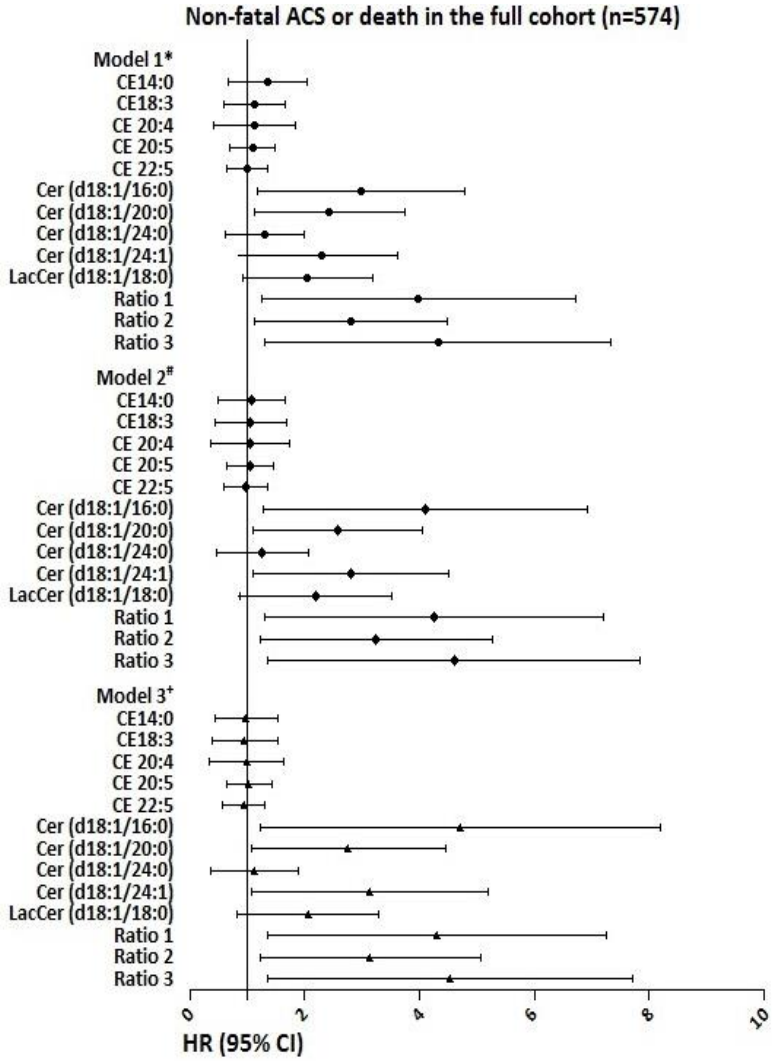
+ Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus, statin use and serum non-HDL cholesterol level at baseline.

Ratio 1:Cer(d18:1/16:0)/Cer(d18:1/24:0)
Ratio 2:Cer(d18:1/20:0)/Cer(d18:1/24:0)
Ratio 3:Cer(d18:1/24:1)/Cer(d18:1/24:0)

Figure 1. Association of plasma concentrations of molecular lipid species with MACE.

The results are presented as hazard ratios (HRs) per unit increase in (Ln-transformed) molecular lipid concentrations or lipid ratios, with 95% confidence intervals (CI).

CE, cholesteryl ester; Cer, ceramide; LacCer, lactosylceramide; MACE, major adverse cardiac events.



* Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus, clinical presentation and statin use.

Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus, clinical presentation, statin use and serum LDL cholesterol level at baseline.

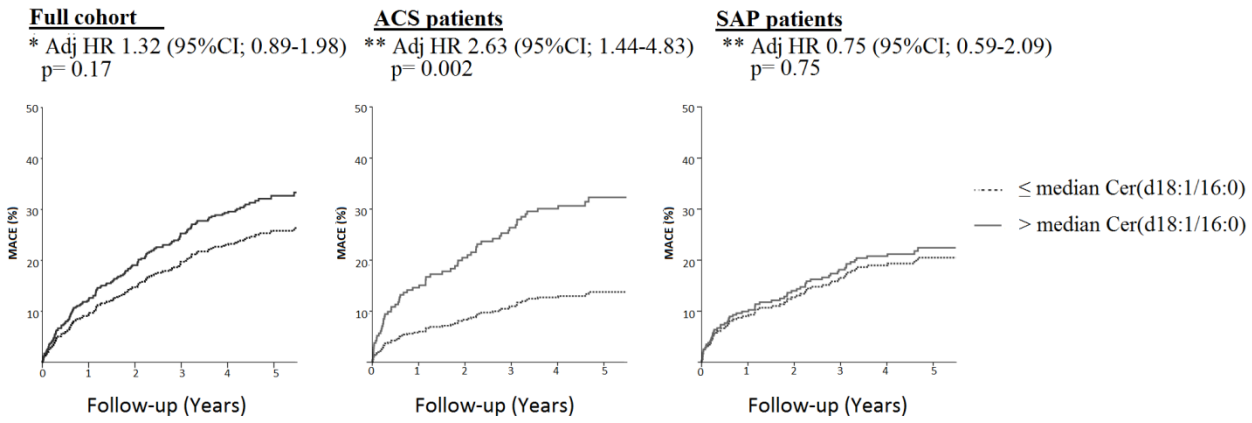
+ Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus, clinical presentation, statin use and serum non-HDL cholesterol level at baseline.

Ratio 1: Cer (d18:1/16:0)/ Cer (d18:1/24:0)
Ratio 2:Cer (d18:1/20:0)/ Cer (d18:1/24:0)
Ratio 3:Cer (d18:1/24:1)/ Cer (d18:1/24:0)

Figure 2. Association of plasma concentrations of molecular lipid species with non-fatal ACS or death.

The results are presented as hazard ratios (HRs) per unit increase in (Ln-transformed) molecular lipid concentrations or lipid ratios, with 95% confidence intervals (CI).

ACS, acute coronary syndrome CE, cholesteryl ester; Cer, ceramide; LacCer, lactosylceramide.



* Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus, clinical presentation, statin use and serum non-HDL cholesterol levels at baseline.
** Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus, statin use and serum non-HDL cholesterol levels at baseline.

Figure 3. Association of plasma concentrations of Cer (d18:1/16:0) with MACE in the full cohort and in patients with ACS or SAP.

The results are presented as Hazard Ratios (HRs) for Cer(d18:1/16:0) above versus below the median, with 95% confidence intervals (CI).

ACS, acute coronary syndrome; Cer, ceramide; MACE, major adverse cardiac events; SAP, stable angina pectoris.