Association between increased plasma ceramides and chronic kidney disease in patients with and without ischemic heart disease

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ABSTRACT

Aim: Plasma levels of certain ceramides are increased in patients with ischemic heart disease (IHD). Many risk factors for IHD are also risk factors for chronic kidney disease (CKD), but it is currently uncertain whether plasma ceramide levels are increased in patients with CKD.

Methods: We measured six previously identified high-risk plasma ceramide concentrations [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 415 individuals who attended our clinical services over a period of 9 months.

Results: 97 patients had CKD (defined as e-$GFR_{CKD\text{-}EPI}$ <60 mL/min/1.73 m^2 and/or urinary albumin-to-creatinine ratio ≥30 mg/g), 117 had established IHD and 242 had type 2 diabetes. Patients with CKD had significantly (p=0.005 or less) higher levels of plasma 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) compared to those without CKD. The presence of CKD remained significantly associated with higher levels of plasma ceramides (standardized beta coefficients ranging from 0.124 to 0.227, p<0.001) even after adjustment for body mass index, smoking, hypertension, diabetes, prior IHD, plasma LDL-cholesterol, hs-C-reactive protein levels and use of any lipid-lowering medications. Notably, more advanced stages of CKD and abnormal albuminuria were both associated (independently of each other) with increased levels of plasma ceramides. These results were consistent in all subgroups considered, including patients with and without established IHD or those with and without diabetes.

Conclusion: Increased levels of plasma ceramides are associated with CKD independently of pre-existing IHD, diabetes and other established cardiovascular risk factors.

Keywords: CKD; ceramides; cardiovascular risk; kidney dysfunction

LIST OF ABBREVIATIONS

ACR, albumin-to-creatinine ratio
BMI, body mass index
Cer, ceramide
CKD, chronic kidney disease
CKD-EPI, chronic kidney disease-epidemiology collaboration
CVD, cardiovascular disease
e-GFR, estimated glomerular filtration rate
ESRD, end-stage renal disease
IHD, ischemic heart disease

INTRODUCTION

Ceramides are highly bioactive lipid molecules composed of a sphingosine backbone attached to a fatty acyl chain. These molecules are found in high concentrations within cell membranes and play important roles as structural lipids and second messengers in both intra-cellular and inter-cellular signaling pathways, regulating apoptosis, proliferation and stress responses [1-3]. Recent prospective studies have shown that increased levels of certain ceramides [mainly plasma Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/20:0) and Cer(d18:1/24:1) levels] are associated with adverse cardiovascular outcomes, independently of traditional risk factors, both in the general adult population and in patients with established ischemic heart disease (IHD) [4-9]. Other studies have shown that higher circulating levels of these ceramides are independently associated with the presence of inducible myocardial ischemia on stress myocardial perfusion scintigraphy [10], or with greater angiographic severity of coronary-artery stenosis in patients with established or suspected IHD [11].

Chronic kidney disease (CKD) has become a public health problem worldwide with an estimated global prevalence of the disease ranging from ~11% to 15% [15]. Notably, CKD is not only associated with an increased risk of developing end-stage renal disease (ESRD) but also cardiovascular disease (CVD) [12-14]. To date, it is uncertain whether there is any association between CKD and circulating levels of specific ceramides that have been associated with an increased risk of incident CVD events [4-9]. The potential existence of an association between increased levels of plasma ceramides and decreased kidney function might therefore contribute, at least in part, to the increased risk of major CVD events observed in patients with CKD.

Therefore, the major aim of this cross-sectional study was to test whether the levels of six previously identified high-risk plasma ceramides [*i.e.*, 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)] were increased in patients with CKD compared to patients without CKD, and whether the relationship between CKD and plasma ceramides was independent of traditional cardiovascular risk factors.

MATERIAL AND METHODS

Patients

For this study, we combined the data from two complementary datasets A & B. (A) 221 patients with established or suspected IHD, who attended the Cardiology service of ''IRCCS Sacro Cuore'' Hospital of Negrar (Verona) over a period of 9 months and performed either a stress myocardial perfusion scintigraphy (*n*=54) or a coronary angiography (*n*=167) for various clinical indications (*e.g.*, chest pain, dyspnea, suspected ischemic electrocardiographic alterations, or echocardiographic abnormalities) [10,11]. (B) 194 patients with established type 2 diabetes attending the Diabetes service of the Azienda Ospedaliera Universitaria Integrata of Verona over a period of 9 months, who underwent imaging tests to diagnose non-alcoholic fatty liver disease (NAFLD) [16]. We excluded patients with a documented history of cirrhosis of any aetiology, active cancer or ESRD (*i.e.*, CKD stage 5, defined as e-GFR <15 mL/min/1.73 m^2 or chronic dialysis).

The local ethics committee approved the study protocol. All participants gave their written informed consent for participation in this study.

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 taking any anti-hypertensive agents.

Venous blood samples were collected in the morning after an overnight fast. Serum creatinine (measured using a Jaffé rate blanked and compensated assay), lipids, high-sensitivity C-reactive protein (hs-CRP that was measured in a subgroup of 377 subjects) and other biochemical parameters were measured by standard laboratory procedures, using the relative reference techniques on Roche Cobas® 8000 (Roche Diagnostics, Basel, Switzerland). Low-density lipoprotein (LDL)-cholesterol was calculated using the Friedewald's equation. Hemoglobin A1c (HbA1c) was measured using the high-performance liquid chromatography analyzer Tosoh-G7 (Tosoh Bioscience Inc., Tokyo, Japan) only in the subgroup of patients with established type 2 diabetes.

e-GFR was estimated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [17]. Urinary albumin excretion rate was assessed with an immuno-nephelometric assay (Beckman-Coulter IMMAGE; Beckman-Coulter Instruments, Fullerton, CA, USA) on a morning spot urine sample and expressed as the albumin-to-creatinine ratio (ACR); abnormal albuminuria was defined as an urinary ACR ≥30 mg/g creatinine (*i.e.*, macroalbuminuria if ACR ≥300 mg/g or microalbuminuria if ACR was between 30 and 299 mg/g). For this study, CKD was defined as the presence of e-$GFR_{CKD\text{-}EPI}$ less than 60 mL/min/1.73 m^2 and/or abnormal albuminuria.

Dyslipidemia was defined as plasma LDL cholesterol >2.60 mmol/L or triglycerides >1.70 mmol/L or use of any lipid-lowering drugs. Presence of diabetes was defined as self-reported physician diagnosis of diabetes, use of anti-hyperglycemic drugs or fasting glucose concentrations ≥7

mmol/L. Presence of IHD was defined as a documented history of acute myocardial infarction, angina pectoris or coronary revascularization procedures.

Plasma ceramides

The methodology for measurement of plasma ceramides has been described previously [10,18]. Briefly, blood samples for ceramide measurements were stored at -80°C until analysis. Previous studies showed that no significant alterations were observed for plasma ceramides upon long-term storage at -80°C [19]. An expert laboratory technician, who was blinded to the clinical details of participants, performed measurement of plasma ceramides for all patients at the central Laboratory of ‘‘IRCCS Sacro Cuore’’ Hospital of Negrar. Ceramides were purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). Plasma 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) concentrations 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). 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]+→264 MRM transition was selected to quantify each ceramide. Both inter-assay and intra-assay coefficients of variations for precision and accuracy for all measured ceramides were less than 15%.

Statistical analysis

Continuous variables were expressed as means±SD or medians (inter-quartile ranges [IQR]) when indicated, whereas categorical variables were expressed as percentages. The Fischer’s exact test

for categorical variables, the Student's *t* test for normally distributed continuous variables or the Mann-Whitney U test for non-normally distributed variables (*i.e*., plasma ceramides, triglycerides, alanine aminotransferase, hs-CRP and e-$GFR_{CKD\text{-}EPI}$ values) were used to examine the differences in clinical and biochemical characteristics as well as plasma ceramides in patients stratified by presence or absence of CKD (defined as e-$GFR_{CKD\text{-}EPI}$ <60 mL/min/1.73 m^2 and/or abnormal albuminuria). The association between CKD and each plasma ceramide (included as the dependent variable in each regression model) was tested using both an univariable linear regression model and a multivariable linear regression model adjusted for BMI, smoking, pre-existing diabetes, hypertension, prior IHD, plasma LDL-cholesterol, plasma hs-CRP, and current use of any anti-hypertensive or lipid-lowering medications. In addition, we also repeated the aforementioned linear regression models, including e-$GFR_{CKD\text{-}EPI}$ values (included as a continuous measure but logarithmically transformed before analysis) and abnormal albuminuria, separately, instead of CKD. Covariates included in these multivariable regression models were selected as potential confounding factors based on their significance in univariable analyses, or their biological plausibility. In these multivariable regression models, we did not include age and sex among the covariates, as these two variables were already included in the equation used for estimating e-$GFR_{CKD\text{-}EPI}$. Subgroup analyses were also performed to assess the association between CKD and each plasma ceramide in patients stratified by sex, age, pre-existing diabetes or established IHD. A *p*-value <0.05 was considered statistically significant. All statistical analyses were performed using the STATA® software, version 14.2.

RESULTS

Of the 415 (259 men and 156 women) patients included in this study, 97 patients had CKD (defined as e-$GFR_{CKD\text{-}EPI}$ <60 ml/min/1.73 m^2 and/or abnormal albuminuria), 242 had established type 2 diabetes and 117 had a previous history of IHD. In particular, with regards to the CKD stages based on e-$GFR_{CKD\text{-}EPI}$ levels (*irrespective* of the presence of abnormal albuminuria), 84.3% (*n*=350) of patients had CKD stages 1 or 2 (*i.e.*, e-$GFR_{CKD\text{-}EPI}$ ≥60 mL/min/1.73 m^2), 11.1% (*n*=46) had CKD stage 3A (e-$GFR_{CKD\text{-}EPI}$ 60-45 mL/min/1.73 m^2), 3.6% (*n*=15) had CKD stage 3B (e-$GFR_{CKD\text{-}EPI}$ 44-30 mL/min/1.73 m^2) and 1% (*n*=4) had CKD stage 4 (e-$GFR_{CKD\text{-}EPI}$ 29-15 mL/min/1.73 m^2). Overall, therefore, 65 (15.7% of total) patients had CKD stage ≥3. By study design, patients with CKD stage 5 (e-$GFR_{CKD\text{-}EPI}$ <15 mL/min/1.73 m^2 or dialysis) were excluded from the analysis. In the whole sample, abnormal albuminuria (*irrespective* of coexisting e-$GFR_{CKD\text{-}EPI}$ levels) was present in 10.6% (*n*=44) of patients (40 of them had microalbuminuria and 4 had macroalbuminuria). In particular, of these 44 patients, 12 patients had also coexisting e-$GFR_{CKD\text{-}EPI}$ <60 mL/min/1.73 m^2, whereas 32 patients had abnormal albuminuria alone.

Table 1 shows the clinical and biochemical characteristics of patients stratified by CKD status. Compared to patients without CKD, those with CKD were older, had higher plasma triglycerides and were more likely to have type 2 diabetes or hypertension. In addition, patients with CKD were also more frequently treated with anti-hyperglycemic agents (*i.e.*, all patients with type 2 diabetes were treated with oral hypoglycemic medications or insulin) or anti-hypertensive drugs, such as angiotensin II receptor blockers, angiotensin-converting enzyme inhibitors or diuretics. The use of anti-platelets, beta-blockers, calcium-channel antagonists or lipid-lowering drugs (statins or fibrates) was essentially comparable between the two patient groups. Furthermore, no significant differences were found in terms of sex distribution, BMI, smoking, plasma lipid profile (except for

triglycerides), glucose parameters (fasting glucose concentrations and HbA1c), hs-CRP, ALT levels, as well as dyslipidemia and prior IHD between the two groups of patients.

As shown in **Figure 1,** patients with CKD had significantly higher levels of all six measured plasma ceramides than those without CKD. Notably, the significant differences between the two groups in all plasma ceramide levels persisted even when we applied a Bonferroni correction for multiple comparisons, using a significance threshold of 0.0083 (*i.e.*, for six ceramides).

As shown in **Figure 2,** patients with CKD also had a significantly higher prevalence of elevated levels of each plasma ceramide (*i.e*., arbitrarily defined as the upper tertile of distribution of each ceramide) compared to patients without CKD.

Figure 3 shows the mean (±SD) levels of plasma ceramides in relation to different stages of CKD based on levels of e-$GFR_{CKD\text{-}EPI}$. Due to very small number of patients with CKD stage 4 (*n*=4), we combined these patients with those belonging to CKD stage 3B. All plasma ceramide levels increased significantly across CKD stages, especially plasma Cer(d18:1/18:0), Cer(d18:1/20:0), and Cer(d18:1/24:1), thus suggesting that these ceramides may be affected by renal clearance.

Table 2 shows the effect of adjustment for multiple cardiometabolic risk factors on the association between CKD and circulating levels of each measured ceramide in the whole sample of patients. Each plasma ceramide was logarithmically transformed and included as the dependent variable in all linear regression models. In univariable regression analyses, presence of CKD was significantly associated with higher levels of plasma 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). All these associations remained statistically

significant even after adjustment for BMI, smoking, pre-existing diabetes, hypertension, prior IHD and use of any lipid-lowering drugs (adjusted model 1). Results remained unchanged even when we additionally adjusted for plasma levels of hs-CRP and LDL-cholesterol (adjusted model 2). We did not further adjust also for age and sex, because these two variables were already included in the e-$GFR_{CKD\text{-}EPI}$ equation. In addition to the presence of CKD, prior IHD, use of lipid-lowering drugs and higher levels of plasma hs-CRP and LDL-cholesterol were also independently associated with each plasma ceramide species in these multivariable linear regression models (data not shown).

Almost similar results were also found when we performed multivariable logistic regression analyses, using the presence of CKD as the dependent variable and including each plasma ceramide as independent variable (**supplementary Table 1**). Each ceramide (expressed for every 1-SD increment) was positively associated with risk of having CKD (with adjusted-odds ratios ranging from 1.54 to 2.12), independently of established risk factors and other potential confounders. The associations between CKD and each plasma ceramide remained statistically significant even when we applied a Bonferroni correction for multiple comparisons (**supplementary Table 1**). Other variables that were independently associated with CKD in these logistic regression models were pre-existing type 2 diabetes and hypertension.

Table 3 shows the effect of adjustment for multiple risk factors on the association between the two individual components of CKD (*i.e.*, decreasing levels of e-$GFR_{CKD\text{-}EPI}$ and abnormal albuminuria) and circulating levels of each ceramide in the whole group of patients. Similarly to what we observed above including the presence of CKD, decreasing e-$GFR_{CKD\text{-}EPI}$ levels were significantly associated with higher plasma Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/20:0) and Cer(d18:1/24:1) levels after adjusting for BMI, smoking, diabetes, hypertension, prior IHD, plasma

LDL-cholesterol, plasma hs-CRP, use of any lipid-lowering drugs and abnormal albuminuria. No independent associations were found between decreasing e-$GFR_{CKD\text{-}EPI}$ values and plasma Cer(d18:1/22:0) or Cer(d18:1/24:0) levels. Conversely, in these multivariable regression models, abnormal albuminuria was independently associated with all plasma ceramides, except for plasma Cer(d18:1/24:1).

Table 4 shows the effect of adjustment for multiple risk factors on the association between the two individual components of CKD (*i.e.*, abnormal albuminuria and four different stages of CKD, based on e-$GFR_{CKD\text{-}EPI}$ values) and plasma ceramides in the whole group of patients. Similar to the observations above, more advanced stages of CKD and abnormal albuminuria were both associated (independently of each other) with increased levels of plasma ceramides.

Finally, we also undertook subgroup analyses by repeating the aforementioned multivariable linear regression analyses in patients stratified by sex, median age), pre-existing type 2 diabetes, or established IHD. The significant and independent associations we observed between CKD and increased levels of each plasma ceramide were consistent in all subgroups considered (data not shown).

DISCUSSION

Presently, there is very limited information about the association between CKD and plasma ceramides. The main findings of our cross-sectional study can be divided into four categories. Firstly, patients with CKD had significantly higher levels of plasma 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)

compared to those without CKD. Secondly, the presence of CKD remained significantly associated with higher levels of these plasma ceramides (each of which have been previously associated with an increased CVD risk), even after adjustment for pre-existing IHD, diabetes, use of any lipid-lowering drugs and other established risk factors. Thirdly, our results were consistent in men and women, in patients with and without pre-existing type 2 diabetes or in those with and without established IHD. Fourthly, when the statistical analyses were repeated using the two individual diagnostic components of CKD separately, both decreasing e-$GFR_{CKD\text{-}EPI}$ levels (or increasing stages of CKD) and abnormal albuminuria were associated, independently of each other, with increased levels of plasma ceramides.

Our results corroborate and expand the findings of two previous small studies [20,21]. In a case-control study involving 93 children with CKD and 24 healthy children, Mitsnefes *et al.* reported that *total* plasma ceramide levels and some their metabolites (such as C24:0 lactosylceramide and C16:0 lactosylceramide) were significantly higher in children with CKD than controls [20]. In another study involving only patients with established type 2 diabetes (126 with abnormal albuminuria, 154 with CKD and 129 without kidney disease), Liu *et al.* showed that circulating levels of sphingomyelin, Cer(18:1/16:0), Cer(18:1/16:1), Cer(18:1/18:0) and sphingosine were significantly higher in diabetic patients with CKD than in their counterparts without kidney disease [21]. The authors also confirmed that decreased e-GFR and abnormal albuminuria were associated with different metabolite signature in these diabetic patients [21].

A number of recent cohort studies have shown that increased levels of certain ceramides are associated with an increased risk of major adverse CVD events, independently of traditional risk factors, both in the general population and in patients with established IHD [4-8]. Many of these

studies were included in a comprehensive meta-analysis that incorporated a total of 7 cohort studies with 29,818 individuals and captured nearly 2,750 major CVD outcomes over a median follow-up of 6 years [9]. This recent meta-analysis concluded that higher levels of plasma Cer(d18:1/16:0), Cer(d18:1/18:0) and Cer(d18:1/24:1) were significantly associated with major adverse CVD events, whereas plasma levels of Cer(d18:1/22:0) and Cer(d18:1/24:0) were not [9]. In our cross-sectional study, we found that patients with CKD had significantly higher levels of all six measured plasma ceramides compared to those without CKD, although the observed inter-group differences appeared to be much greater for plasma Cer(d18:1/18:0), Cer(d18:1/20:0) and Cer(d18:1/24:1) levels. As shown in **Figure 3**, the levels of plasma Cer(d18:1/18:0), Cer(d18:1/20:0) and Cer(d18:1/24:1) were those that more markedly increased across CKD stages, thus suggesting that these ceramides can be affected by renal clearance. It is, therefore, possible to hypothesize that there are more similarities than differences in the etiopathogenetic mechanisms leading to the development of CVD and CKD. However, prospective studies are needed to confirm that increased levels of the aforementioned plasma ceramides are associated not only with an increased risk of major CVD events but also with an increased risk of developing CKD. Further research is also required to better elucidate the different effects of plasma ceramides with various acyl-chain lengths and also with different saturated/ unsaturated compounds on signaling pathways involved in CVD and CKD development.

To date, the biological mechanisms underlying the association between CKD and increased levels of plasma ceramides are poorly understood. It should be noted that the associations we observed between CKD and increased plasma ceramides remained statistically significant even after adjustment for multiple cardiometabolic risk factors, including BMI, smoking, hypertension, pre-existing diabetes, prior IHD, plasma LDL-cholesterol, plasma hs-CRP and use of lipid-lowering or

anti-hypertensive medications. These results were also consistent in all subgroups considered. Hence, it is reasonable to assume that other underlying mechanisms may be involved in the link between CKD and increased plasma ceramides. It is well known that CKD is associated with perturbations in lipoprotein metabolism, thereby leading to atherogenic dyslipidemia [22]. Interestingly, experimental findings now suggest perturbations in other lipid species, including sphingolipids, in CKD [23,24]. Furthermore, strong evidence indicates that patients with CKD have higher pro-inflammatory and pro-oxidant factors compared with non-CKD patients [12-14]. Experimentally, it has been shown that these pro-inflammatory and pro-oxidant factors may activate sphingomyelinase and other enzymes directly implicated in the production of ceramide species [3,24,25]. On the other hand, it is known that ceramides play important roles in the regulation of inflammatory responses [26-28]. Emerging evidence is also suggesting that sphingolipids, including certain ceramides, may play a role in the pathogenesis and progression of genetic and non-genetic forms of CKD [24,29]. However, further mechanistic studies are needed to better understand the complex and intertwined links between plasma ceramides and risk of CKD.

Further research is required to elucidate the different effects of plasma ceramides with various acyl-chain lengths and also with different saturated/ unsaturated compounds on signaling pathways involved in cardiovascular disease.

Our study has some important limitations that should be mentioned. Firstly, the cross-sectional design of our study does not allow establishing temporality and causality of the observed associations. In fact, based on the available published evidence to date, it is plausible that CKD might lead to elevated plasma ceramides and vice versa. Secondly, in this study we have recruited a large number of patients at relatively high CVD risk. Hence, our results might not be generalizable to other cohorts of patients at low or intermediate CVD risk. Thirdly, we cannot rule

out the possibility that other unmeasured factors could partly explain the observed associations. Finally, we did not measure other biomarkers in the ceramide pathway (*e.g.*, sphingomyelins).

Despite these limitations, our study has also a number of important strengths, such as the large sample size, the consecutive enrolment of patients, the completeness of database, the adjustment for multiple established cardiometabolic risk factors, and the exclusion of patients with active cancer, decompensated cirrhosis or ESRD (*i.e.*, CKD stage 5). We believe that the inclusion of patients with such serious co-morbidities could confound the interpretation of data when studying relationships between plasma ceramides and outcomes.

We believe that the results of our study are clinically important, because they clearly highlight that CKD may adversely affect circulating levels of long- and very long-chain ceramides, explaining at least in part the increased risk of CVD observed among patients with CKD [12-14]. Notably, more advanced stages of CKD and abnormal albuminuria are both associated (independently of each other) with increased plasma ceramides. These results also emphasize that parameters of kidney function should be always measured in cohort studies examining the role of plasma ceramides in predicting risk of CVD events and mortality.

In conclusion, the results of this cross-sectional study show that the presence of CKD is strongly associated with circulating levels of six previously identified plasma ceramides. These associations were observed regardless of the presence of pre-existing IHD, diabetes, plasma LDL-cholesterol levels, use of lipid-lowering drugs and other established risk factors. However, the cross-sectional design of the study does not allow for proving any cause-and-effect relationship. Further research

is now needed to confirm these results in independent samples and better elucidate the mechanisms underlying the observed associations.

Disclosure Statement: All authors declare no conflicts of interest.

Authors' contributions: AM and GT conceived and designed the study. GL measured plasma ceramides. GL, AM, SB, CD, AA, GM, AC and CB researched data and reviewed/edited the manuscript. GL, SB, RL, FB and CDB contributed to discussion and reviewed/edited the manuscript. AM and GT analyzed the data and wrote the manuscript. AM and GT are the guarantors of this work and, as such, had full access to all the data of the study and take responsibility for the integrity and accuracy of data. All authors approved the final version of the manuscript.

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Table 1. Clinical and biochemical characteristics of patients stratified by presence or absence of chronic kidney disease (CKD).

	All patients (*n*=415)	**Without CKD (*n*=318)**	**With CKD (*n*=97)**	***P* value**
Age (years)	69.7 ± 10	68.8 ± 10	72.4 ± 9	0.002
Sex (male) (%)	62.4	61.3	65.9	0.473
BMI (kg/m^2)	27.6 ± 4	27.4 ± 4	28.2 ± 5	0.167
Current smokers (%)	12.5	12.6	12.4	0.978
Systolic blood pressure (mmHg)	136 ± 17	135 ± 18	136 ± 16	0.737
Diastolic blood pressure (mmHg)	76 ± 10	76 ± 9	76 ± 10	0.917
Total cholesterol (mmol/L)	4.21 ± 1.0	4.20 ± 1.0	4.12 ± 1.1	0.478
LDL cholesterol (mmol/L)	2.30 ± 0.9	2.30 ± 0.9	2.23 ± 0.9	0.334
HDL cholesterol (mmol/L)	1.31 ± 0.3	1.30 ± 0.4	1.21 ± 0.3	0.070
Triglycerides (mmol/L)	1.20 (0.9-1.7)	1.11 (0.9-1.6)	1.51 (1.1-2.1)	<0.001
Fasting glucose (mmol/L)	6.8 ± 2.0	6.7 ± 1.9	7.1 ± 2.3	0.092
HbA1c (mmol/mol), *n*=242*	50.5 ± 12	50.0 ± 11	51.6 ± 13	0.352
hs-CRP (mg/L), *n*=377	1.8 (0.9-4.0)	1.6 (1.1-3.8)	2.1 (1.0-5.4)	0.368
ALT (IU/L)	15 (11-22)	15 (11-23)	14 (10-19)	0.491
Creatinine (µmol/L)	80.9 ± 32	72.9 ± 15	107.3 ± 53	<0.001
e-GFR$_{CKD-EPI}$ (mL/min/1.73 m^2)	81.5 (66.7-92.1)	85.9 (73.8-93.2)	55.2 (46.6-71.5)	<0.001
Abnormal albuminuria	10.6	0.0	46.4	<0.001
Type 2 diabetes (%)	58.3	53.5	74.2	<0.001
Dyslipidemia (%)	84.6	83.6	87.6	0.217
Hypertension (%)	80.9	77.9	90.7	0.003
Prior IHD (%)	28.3	27.4	30.9	0.293
Antihyperglycemic agents (%)	58.3	53.5	74.2	0.008
Anti-platelets (%)	53.4	52.1	57.7	0.194
Beta-blockers (%)	40.5	39.7	43.3	0.302
ARBs/ACE-inhibitors (%)	62.3	57.9	76.3	<0.001
Calcium-channel antagonists (%)	20.4	19.4	23.7	0.215
Diuretics (%)	32.8	27.3	50.5	<0.001
Statins (%)	62.4	61.0	67.1	0.256
Fibrates (%)	3.2	3.2	3.0	0.634

Sample size, *n*=415, except where indicated. Data are expressed as means± SD, medians (IQRs) or percentages. Differences between the two groups were tested by the Fischer's exact test for categorical variables, the Student's *t* test for normally distributed variables, and the Mann-Whitney U test for non-normally distributed variables (*i.e.*, plasma triglycerides, hs-CRP, ALT and e-GFR$_{CKD-EPI}$). *HbA1c level was measured only in patients with established diabetes.

Note: CKD was defined as e-GFR$_{CKD-EPI}$ <60 mL/min/1.73 m^2 and/or abnormal albuminuria. Abnormal albuminuria was defined as urinary ACR ≥30 mg/g creatinine. Dyslipidemia was defined as LDL cholesterol >2.60 mmol/L or triglycerides >1.70 mmol/L or use of any lipid-lowering agents. Hypertension was defined as blood pressure ≥140/90 mmHg or use of any anti-hypertensive drugs.

Abbreviations: ACE, angiotensin-converting enzyme; ALT, alanine aminotransferase; ARB, angiotensin II receptor blocker; BMI, body mass index; e-GFR$_{CKD-EPI}$, estimated glomerular filtration rate (assessed by the CKD-EPI equation); hs-CRP, high sensitivity C-reactive protein; IHD, ischemic heart disease.

Table 2. Unadjusted and adjusted associations of each plasma ceramide with presence of CKD.

Linear Regression Models	Standardized beta coefficient(s)	*P* value
Log Cer(d18:1/16:0) (µmol/L)		
CKD (yes vs. no)		
Unadjusted model	0.198	<0.001
Adjusted model 1*	0.232	<0.001
Adjusted model 2§ (*n*=377)	0.224	<0.001
Log Cer(d18:1/18:0) (µmol/L)		
CKD (yes vs. no)		
Unadjusted model	0.189	<0.001
Adjusted model 1*	0.201	<0.001
Adjusted model 2§ (*n*=377)	0.180	0.001
Log Cer(d18:1/20:0) (µmol/L)		
CKD (yes vs. no)		
Unadjusted model	0.216	<0.001
Adjusted model 1*	0.236	<0.001
Adjusted model 2§ (*n*=377)	0.227	<0.001
Log Cer(d18:1/22:0) (µmol/L)		
CKD (yes vs. no)		
Unadjusted model	0.141	0.004
Adjusted model 1*	0.162	0.001
Adjusted model 2§ (*n*=377)	0.171	0.001
Log Cer(d18:1/24:0) (µmol/L)		
CKD (yes vs. no)		
Unadjusted model	0.117	0.017
Adjusted model 1*	0.119	0.013
Adjusted model 2§ (*n*=377)	0.124	0.011
Log Cer(d18:1/24:1) (µmol/L)		
CKD (yes vs. no)		
Unadjusted model	0.212	<0.001
Adjusted model 1*	0.240	<0.001
Adjusted model 2§ (*n*=377)	0.218	<0.001

Sample size, *n*=415, unless otherwise indicated. Data are expressed as standardized beta coefficients as tested by univariable (unadjusted) and multivariable (adjusted) linear regression models. Each plasma ceramide was logarithmically transformed (Log) and included as the dependent variable in all linear regression models. Chronic kidney disease (CKD) was defined as e-$GFR_{CKD\text{-}EPI}$ <60 mL/min/1.73 m^2 and/or abnormal albuminuria (urinary ACR ≥30 mg/g creatinine).

* Other covariates that were simultaneously included in multivariable linear regression model 1 (along with CKD) were as follows: BMI, smoking history, pre-existing type 2 diabetes, hypertension (i.e., blood pressure ≥140/90 mmHg or drug treatment), prior IHD, and use of lipid-lowering drugs (i.e., statins or fibrates).

§ In adjusted regression model 2 (performed in a sample of 377 patients with available data for plasma hs-CRP levels), we included the same covariates of model 1 *plus* plasma LDL-cholesterol and hs-CRP levels.

Table 3. Adjusted associations of each plasma ceramide with decreasing levels of e-$GFR_{CKD\text{-}EPI}$ (included as a continuous measure) and abnormal albuminuria.

Multivariable Linear Regression Models	Standardized beta coefficient(s)	*P* value
Log Cer(d18:1/16:0) (µmol/L)		
Log e-$GFR_{CKD\text{-}EPI}$ (mL/min/1.73 m^2)	-0.133	0.008
Abnormal albuminuria (yes vs. no)	0.173	0.001
BMI (kg/m^2)	-0.148	0.003
Smoking status (yes vs. no)	0.009	0.834
Diabetes (yes vs. no)	0.081	0.156
Hypertension (yes vs. no)	0.003	0.953
Prior IHD (yes vs. no)	-0.088	0.102
Lipid-lowering drug users (yes vs. no)	-0.160	0.003
LDL-cholesterol (mmol/L)	0.297	<0.001
hs-CRP (mg/L)	0.106	0.034
Log Cer(d18:1/18:0) (µmol/L)		
Log e-$GFR_{CKD\text{-}EPI}$ (mL/min/1.73 m^2)	-0.138	0.009
Abnormal albuminuria (yes vs. no)	0.128	0.017
BMI (kg/m^2)	0.004	0.936
Smoking status (yes vs. no)	0.001	0.992
Diabetes (yes vs. no)	0.013	0.833
Hypertension (yes vs. no)	0.032	0.568
Prior IHD (yes vs. no)	-0.089	0.125
Lipid-lowering drug users (yes vs. no)	-0.039	0.498
LDL-cholesterol (mmol/L)	0.139	0.019
hs-CRP (mg/L)	0.181	0.001
Log Cer(d18:1/20:0) (µmol/L)		
Log e-$GFR_{CKD\text{-}EPI}$ (mL/min/1.73 m^2)	-0.147	0.006
Abnormal albuminuria (yes vs. no)	0.124	0.020
BMI (kg/m^2)	-0.078	0.147
Smoking status (yes vs. no)	0.002	0.971
Diabetes (yes vs. no)	-0.003	0.955
Hypertension (yes vs. no)	0.041	0.456
Prior IHD (yes vs. no)	-0.100	0.079
Lipid-lowering drug users (yes vs. no)	0.052	0.371
LDL-cholesterol (mmol/L)	0.220	<0.001
hs-CRP (mg/L)	0.146	0.006
Log Cer(d18:1/22:0) (µmol/L)		
Log e-$GFR_{CKD\text{-}EPI}$ (mL/min/1.73 m^2)	0.042	0.400
Abnormal albuminuria (yes vs. no)	0.145	0.004
BMI (kg/m^2)	0.011	0.820
Smoking status (yes vs. no)	0.009	0.848
Diabetes (yes vs. no)	0.126	0.030
Hypertension (yes vs. no)	-0.093	0.069
Prior IHD (yes vs. no)	-0.099	0.066
Lipid-lowering drug users (yes vs. no)	0.003	0.956

LDL-cholesterol (mmol/L)	0.384	<0.001
hs-CRP (mg/L)	0.132	0.009
Log Cer(d18:1/24:0) (µmol/L)		
Log e-$GFR_{CKD-EPI}$ (mL/min/1.73 m^2)	0.010	0.841
Abnormal albuminuria (yes vs. no)	0.138	0.005
BMI (kg/m^2)	-0.048	0.318
Smoking status (yes vs. no)	-0.002	0.971
Diabetes (yes vs. no)	0.253	<0.001
Hypertension (yes vs. no)	-0.117	0.018
Prior IHD (yes vs. no)	-0.145	0.006
Lipid-lowering drug users (yes vs. no)	0.183	0.001
LDL-cholesterol (mmol/L)	0.418	<0.001
hs-CRP (mg/L)	0.064	0.190
Log Cer(d18:1/24:1) (µmol/L)		
Log e-$GFR_{CKD-EPI}$ (mL/min/1.73 m^2)	-0.183	0.001
Abnormal albuminuria (yes vs. no)	0.090	0.085
BMI (kg/m^2)	-0.058	0.260
Smoking status (yes vs. no)	-0.053	0.276
Diabetes (yes vs. no)	-0.029	0.622
Hypertension (yes vs. no)	0.047	0.373
Prior IHD (yes vs. no)	-0.091	0.108
Lipid-lowering drug users (yes vs. no)	-0.089	0.107
LDL-cholesterol (mmol/L)	0.236	<0.001
hs-CRP (mg/L)	0.138	0.008

Sample size, *n*=377 because plasma hs-CRP level was available in 377 patients. Data are expressed as standardized beta coefficients as tested by multivariable linear regression analysis. Each ceramide was logarithmically transformed (Log) and included as the dependent variable in all linear regression models. e-$GFR_{CKD-EPI}$ levels were included as a continuous measure and were logarithmically transformed before analysis. Abnormal albuminuria was defined as urinary ACR ≥30 mg/g creatinine.

Other covariates that were simultaneously included in these six multivariable linear regression models along with e-$GFR_{CKD-EPI}$ levels and abnormal albuminuria were as follows: BMI, smoking, pre-existing type 2 diabetes, hypertension (i.e., blood pressure ≥140/90 mmHg or drug treatment), prior IHD, plasma LDL-cholesterol level, plasma hs-CRP level and use of lipid-lowering drugs (i.e., statins or fibrates).

Abbreviations: BMI, body mass index; e-$GFR_{CKD-EPI}$, estimated glomerular filtration rate (assessed by the CKD-EPI equation); hs-CRP, high sensitivity C-reactive protein; IHD, ischemic heart disease.

Table 4 Adjusted associations of each plasma ceramide with different stages of CKD (based on e-$GFR_{CKD\text{-}EPI}$ levels) and abnormal albuminuria.

Multivariable Linear Regression Models	Standardized beta coefficient(s)	*P* value
Log Cer(d18:1/16:0) (µmol/L)		
CKD stages		
Stage 1 (≥90 mL/min/1.73 m^2)	Ref.	-
Stage 2 (89-60 mL/min/1.73 m^2)	0.023	0.680
Stage 3A (59-45 mL/min/1.73 m^2)	0.108	0.043
Stages 3B+4 (<45 mL/min/1.73 m^2)	0.116	0.027
Abnormal albuminuria (yes vs. no)	0.165	0.001
BMI (kg/m^2)	-0.144	0.005
Smoking status (yes vs. no)	0.020	0.686
Diabetes (yes vs. no)	0.109	0.066
Hypertension (yes vs. no)	0.006	0.907
Prior IHD (yes vs. no)	-0.088	0.106
Lipid-lowering drug users (yes vs. no)	-0.112	0.045
LDL-cholesterol (mmol/L)	0.351	<0.001
hs-CRP (mg/L)	0.111	0.026
Log Cer(d18:1/18:0) (µmol/L)		
CKD stages		
Stage 1 (≥90 mL/min/1.73 m^2)	Ref.	-
Stage 2 (89-60 mL/min/1.73 m^2)	0.022	0.720
Stage 3A (59-45 mL/min/1.73 m^2)	0.058	0.308
Stages 3B+4 (<45 mL/min/1.73 m^2)	0.139	0.013
Abnormal albuminuria (yes vs. no)	0.119	0.029
BMI (kg/m^2)	0.015	0.978
Smoking status (yes vs. no)	0.015	0.781
Diabetes (yes vs. no)	0.082	0.199
Hypertension (yes vs. no)	0.020	0.719
Prior IHD (yes vs. no)	-0.088	0.134
Lipid-lowering drug users (yes vs. no)	0.011	0.858
LDL-cholesterol (mmol/L)	0.201	0.001
hs-CRP (mg/L)	0.180	0.001
Log Cer(d18:1/20:0) (µmol/L)		
CKD stages		
Stage 1 (≥90 mL/min/1.73 m^2)	Ref.	-
Stage 2 (89-60 mL/min/1.73 m^2)	0.049	0.411
Stage 3A (59-45 mL/min/1.73 m^2)	0.133	0.020
Stages 3B+4 (<45 mL/min/1.73 m^2)	0.150	0.007
Abnormal albuminuria (yes vs. no)	0.113	0.035
BMI (kg/m^2)	-0.069	0.202
Smoking status (yes vs. no)	0.003	0.950
Diabetes (yes vs. no)	0.038	0.551
Hypertension (yes vs. no)	0.028	0.618
Prior IHD (yes vs. no)	-0.102	0.078

Lipid-lowering drug users (yes vs. no)	0.106	0.073
LDL-cholesterol (mmol/L)	0.281	<0.001
hs-CRP (mg/L)	0.149	0.005
Log Cer(d18:1/22:0) (µmol/L)		
CKD stages		
Stage 1 (≥90 mL/min/1.73 m^2)	Ref.	-
Stage 2 (89-60 mL/min/1.73 m^2)	-0.045	0.418
Stage 3A (59-45 mL/min/1.73 m^2)	0.051	0.334
Stages 3B+4 (<45 mL/min/1.73 m^2)	0.058	0.267
Abnormal albuminuria (yes vs. no)	0.134	0.008
BMI (kg/m^2)	0.011	0.834
Smoking status (yes vs. no)	0.012	0.796
Diabetes (yes vs. no)	0.145	0.015
Hypertension (yes vs. no)	-0.099	0.059
Prior IHD (yes vs. no)	-0.097	0.075
Lipid-lowering drug users (yes vs. no)	0.048	0.389
LDL-cholesterol (mmol/L)	0.421	<0.001
hs-CRP (mg/L)	0.134	0.008
Log Cer(d18:1/24:0) (µmol/L)		
CKD stages		
Stage 1 (≥90 mL/min/1.73 m^2)	Ref.	-
Stage 2 (89-60 mL/min/1.73 m^2)	-0.050	0.368
Stage 3A (59-45 mL/min/1.73 m^2)	-0.010	0.867
Stages 3B+4 (<45 mL/min/1.73 m^2)	0.045	0.371
Abnormal albuminuria (yes vs. no)	0.130	0.009
BMI (kg/m^2)	-0.041	0.418
Smoking status (yes vs. no)	-0.009	0.853
Diabetes (yes vs. no)	0.253	<0.001
Hypertension (yes vs. no)	-0.121	0.019
Prior IHD (yes vs. no)	-0.142	0.008
Lipid-lowering drug users (yes vs. no)	0.211	<0.001
LDL-cholesterol (mmol/L)	0.440	<0.001
hs-CRP (mg/L)	0.059	0.228
Log Cer(d18:1/24:1) (µmol/L)		
CKD stages		

Stage 1 (≥90 mL/min/1.73 m^2)	Ref.	-
Stage 2 (89-60 mL/min/1.73 m^2)	0.018	0.754
Stage 3A (59-45 mL/min/1.73 m^2)	0.160	0.004
Stages 3B+4 (<45 mL/min/1.73 m^2)	0.171	0.002
Abnormal albuminuria (yes vs. no)	0.075	0.151
BMI (kg/m^2)	-0.053	0.317
Smoking status (yes vs. no)	-0.093	0.074
Diabetes (yes vs. no)	0.010	0.873
Hypertension (yes vs. no)	0.041	0.456
Prior IHD (yes vs. no)	-0.089	0.113
Lipid-lowering drug users (yes vs. no)	-0.044	0.445
LDL-cholesterol (mmol/L)	0.264	<0.001
hs-CRP (mg/L)	0.143	0.006

Sample size, *n*=377 because plasma hs-CRP level was available in 377 patients. Data are expressed as standardized beta coefficients as tested by multivariable linear regression analysis. Each ceramide was logarithmically transformed (Log) and included as the dependent variable in all linear regression models. Stages of CKD were defined based on the levels of e-$GFR_{CKD\text{-}EPI}$. Abnormal albuminuria was defined as urinary ACR ≥30 mg/g creatinine.

Other covariates that were simultaneously included in these six multivariable linear regression models along with four CKD stages (based on e-GFR levels) and abnormal albuminuria were as follows: BMI, smoking, pre-existing type 2 diabetes, hypertension (i.e., blood pressure ≥140/90 mmHg or drug treatment), prior IHD, plasma LDL-cholesterol level, plasma hs-CRP level and use of lipid-lowering drugs (i.e., statins or fibrates).

Abbreviations: BMI, body mass index; hs-CRP, high sensitivity C-reactive protein; IHD, ischemic heart disease.

FIGURE LEGENDS

Figure 1. Box plots of six measured plasma ceramide levels in 415 patients stratified by presence or absence of CKD (defined as e-$GFR_{CKD\text{-}EPI}$ <60 mL/min/1.73 m^2 and/or urinary ACR ≥30 mg/g creatinine). In each panel, differences between the two groups of patients were tested by the Mann-Whitney U test. The central rectangle spans the 1st quartile to the 3rd quartile (i.e., the inter-quartile range [IQR]). The segment inside the rectangle shows the median and the ''whiskers'' above and below the box show the locations of 1.5 x IQR values.

Figure 2. Prevalence of abnormal levels of each plasma ceramide (i.e., arbitrarily defined as the upper tertile of distribution of each ceramide) in 415 patients stratified by presence or absence of CKD (defined as e-$GFR_{CKD\text{-}EPI}$ <60 mL/min/1.73 m^2 and/or urinary ACR ≥30 mg/g creatinine). In each panel, differences between the two groups of patients were tested by the Fischer's exact test. The cutoff values of the upper tertile of distribution of each plasma ceramide were as follows: >0.346 μmol/L for Cer(d18:1/16:0), >0.137 μmol/L for Cer(d18:1/18:0), >0.112 μmol/L for Cer(d18:1/20:0), >0.833 μmol/L for Cer(d18:1/22:0), >3.475 μmol/L for Cer(d18:1/24:0) and >1.153 μmol/L for Cer(d18:1/24:1), respectively.

Figure 3. Mean±SD levels of plasma ceramides in relation to increasing stages of CKD [stage 1 (*n*=134): e-$GFR_{CKD\text{-}EPI}$ ≥90 mL/min/1.73 m^2; stage 2 (*n*=216): e-$GFR_{CKD\text{-}EPI}$ between 89 and 60 mL/min/1.73 m^2; stage 3A (*n*=46): e-$GFR_{CKD\text{-}EPI}$ between 59 and 45 mL/min/1.73 m^2; stages 3B+4 (*n*=19): e-$GFR_{CKD\text{-}EPI}$ <44 mL/min/1.73 m^2]. In each panel, *P*-values for trends were tested by the Kruskal-Wallis test (the Dunn's test was used for multiple pairwise comparisons).