ORIGINAL RESEARCH ARTICLE

Relation between plasma ceramides and cardiovascular death in chronic heart failure: A subset analysis of the GISSI-HF trial

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Abstract

Aims Ceramides exert several biological activities that may contribute to the pathophysiology of cardiovascular disease and heart failure (HF). The association between plasma levels of distinct ceramides (that have been previously associated with increased cardiovascular risk) and cardiovascular mortality in patients with chronic HF has received little attention.

Methods and results In a post hoc ancillary analysis of the Gruppo Italiano per lo Studio della Sopravvivenza nella Insufficienza Cardiaca-Heart Failure (GISSI-HF; NCT00336336) trial, we randomly selected a sample of 200 ambulatory patients with chronic HF who died due to cardiovascular causes and 200 patients who were alive at the end of the trial (after a median follow-up period of 3.9 years). We measured baseline plasma concentrations of six previously identified high-risk 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) and their individual plasma ratios with Cer(d18:1/24:0)]. Patients who died due to cardiovascular causes had significantly ($P < 0.05$ or less) higher levels of plasma Cer(d18:1/16:0) and Cer(d18:1/24:1), but lower levels of plasma Cer(d18:1/22:0) and Cer(d18:1/24:0) than had those who did not. All plasma ratios of each ceramide with Cer(d18:1/24:0) were significantly higher in patients who died due to cardiovascular causes. In Cox regression analyses, all five plasma ratios of each ceramide with Cer(d18:1/24:0) were significantly associated with a greater risk of cardiovascular mortality (with unadjusted hazard ratios ranging from 1.23 to 1.59; $P < 0.001$ or less). These significant associations were attenuated after adjustment for multiple established risk factors, New York Heart Association functional class, left ventricular ejection fraction, use of medications, plasma pentraxin-3 levels, and, especially, plasma N-terminal pro-brain natriuretic peptide (NT-proBNP) levels. When we applied a Bonferroni correction for multiple comparisons (using a P-threshold 0.05/5 ceramide ratios = 0.01), none of the five plasma ratios of each ceramide with Cer(d18:1/24:0) remained statistically associated with the risk of cardiovascular mortality (with adjusted hazard ratios ranging from 1.10 to 1.23).

Conclusions Higher levels of specific plasma ceramides [especially when used in ratios with Cer(d18:1/24:0)] are associated with increased cardiovascular mortality in ambulatory patients with chronic HF. However, these associations are weakened after adjustment for established cardiovascular risk factors, medication use, and plasma NT-proBNP concentrations.

Introduction

Ceramides are highly bioactive lipid molecules composed of a sphingosine backbone attached to a fatty acyl chain that are found in high concentrations within cell membranes and play important roles as both structural lipids and second messengers for intra-cellular and inter-cellular signalling pathways, such as cellular growth, proliferation, differentiation, and apoptosis.[1,2] Over the last decade, convincing evidence mainly derived from *in vitro* experiments and *in vivo* animal studies has supported the importance of specific ceramides as possible mediators or biomarkers of complex disease mechanisms, including atherosclerotic processes.[1,3,4]

Large prospective studies have recently identified plasma ceramides with long and very-long chains [mostly increased plasma levels of Cer(d18:1/16:0), Cer(d18:1/18:0), and Cer (d18:1/24:1) and their ratios with Cer(d18:1/24:0)] as significant predictors of cardiovascular morbidity and mortality both in the general population[5] and in patients with established coronary artery disease (CAD) or acute coronary syndrome, independent of traditional cardiovascular risk factors.[6–9] Additionally, higher levels of these plasma ceramides have been strongly associated with greater angiographic severity of coronary stenoses in patients with CAD,[10] as well as with lower post-stress myocardial wall perfusion in patients with established, or suspected, CAD undergoing stress myocardial perfusion scintigraphy.[11]

That said, in this hypothesis-generating study, we have supposed that there is a significant association between higher plasma levels of specific ceramides (that have been previously associated with higher risk of adverse cardiovascular outcomes in patients with CAD) and the risk of cardiovascular mortality also in patients with chronic heart failure (HF), that is, a progressive, complex clinical syndrome characterized by high rates of cardiovascular morbidity and mortality.[12,13] However, to the best of our knowledge, no evidence is currently available for the existence of a significant association between the aforementioned plasma ceramides [and their plasma ratios with Cer(d18:1/24:0)] and the risk of cardiovascular mortality among patients with chronic HF. We believe that this topic is of clinical importance, because the observation of an association between circulating levels of specific plasma ceramides and risk of cardiovascular mortality in this patient population might lead to improved pathophysiologic understanding of the complex biological mechanisms promoting cardiovascular mortality/morbidity in chronic HF and might also provide new potential targets for management of CAD in this group of patients.

Therefore, in this post hoc ancillary analysis of the Gruppo Italiano per lo Studio della Sopravvivenza nella Insufficienza Cardiaca-Heart Failure (GISSI-HF) trial, we aimed at examining whether there were significant associations between circulating levels of previously identified high-risk plasma ceramides [and their plasma ratios with Cer(d18:1/24:0)] and the risk of cardiovascular mortality among ambulatory patients with chronic HF.

Methods

Study population and design

The design and the main results of the GISSI-HF trial have been thoroughly presented elsewhere in prior publications,[14,15] and the registered study protocol is available online at https://clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT00336336).

Briefly, the GISSI-HF trial started in 2002 as a pragmatic, double-blind, placebo-controlled, nationwide multicentre study in 326 cardiology and 31 internal medicine centres with the specific aim of testing with a nested design the efficacy and safety of two different drugs [i.e. rosuvastatin 10 mg/day or *n*−3 polyunsaturated fatty acids (*n*−3 PUFAs) 1 g/day] in ambulatory patients with chronic HF. The trial enrolled 6975 patients with clinical evidence of chronic and stable HF [New York Heart Association (NYHA) functional class II–IV], irrespective of age, left ventricular (LV) ejection fraction, and HF aetiology. Major exclusion criteria included the presence of any non-cardiac co-morbidity that was unlikely to be compatible with sufficiently long follow-up (e.g. active cancer); acute coronary syndrome or revascularization procedures within 1 month; planned cardiovascular surgery, expected to be performed within 3 months after randomization; significant liver diseases; serum creatinine concentrations $\geq$ 2.5 mg/dL; and serum aminotransferase concentrations > 1.5 times the upper normal limit.[14,15]

Primary endpoint of the GISSI-HF trial was time to death, and time to death or admission to hospital for cardiovascular causes. Over the follow-up period of the trial (median duration of 3.9 years), there were 1969 total deaths.[14,15] Among the specific causes of death, ~75% were cardiovascular (mainly due to worsening HF or sudden cardiac death), ~21% were non-cardiovascular (mainly due to malignancy or acute infections), and ~4% were unknown. All of these study endpoints were adjudicated blindly by an *ad hoc* committee on the basis of pre-agreed definitions and procedures.[14,15]

In the present post-hoc ancillary analysis of the GISSI-HF trial, using the stored blood samples of participants enrolled in the 51 clinical centres adhering to the biologic sub-study, we have randomly selected a sample of 200 patients with chronic HF who died due to cardiovascular causes (i.e. cases) and 200 patients who were alive at the end of the trial (i.e. controls).

The GISSI-HF trial was approved by each local Institutional Review Board of all the participating centres. A written informed consent was obtained from each study participant before the study enrolment.

Plasma ceramides

The methodology for measurement of plasma ceramides has been described previously.[11,17] 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.[16] An expert laboratory technician (G. L.), who was blinded to the clinical details of the participants, performed all measurements of plasma ceramides at the central Laboratory of the 'IRCCS Sacro Cuore' Hospital of Negrar. Ceramide standards were purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). Plasma concentrations of Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/20:0, Cer(d18:1/22:0), Cer(d18:1/24:0), and Cer(d18:1/24:1) were measured by liquid–liquid extraction with 2-propanol:ethyl acetate (4:1 v/v) and gradient reverse-phased chromatography on an Agilent Poroshell 120 C18 column (4.6 × 50 mm, 2.7 μm).[11,17] Cer(d18:1/17:0) was used as internal standard. The apparatus consisted of an Agilent 1290 ultra-high-performance liquid chromatography system coupled with an Agilent 6495 Triple Quadrupole liquid chromatography/mass spectrometry (LC/MS) system. Mobile phases consisted in LC/MS grade water, acetonitrile with 0.1% formic acid, and 10 mM ammonium acetate in 2-propanol. $[M + H] + \rightarrow 264$ MRM (multiple reaction monitoring) transition was selected to quantify each ceramide. Calibration standards (six points) were prepared each day in surrogate matrix (5% bovine serum albumin) at concentrations ranging from 1.0 to 0.031 μM/L for Cer(d18:1/16:0), Cer(d18:1/18:0), and Cer(d18:1/20:0) and from 10 to 0.31 μM/L for Cer(d18:1/22:0), Cer(d18:1/24:0), and Cer(d18:1/24:1), respectively. Linearity regression coefficients were $R^2 > 0.99$ for all ceramides. The inter-assay and intra-assay coefficients of variation for precision and accuracy for all measured ceramides were <15%.[11,17] Each plasma ceramide was expressed both as crude concentration and as ratio with Cer(d18:1/24:0). In particular, the ratios of each plasma ceramide with Cer(d18:1/24:0) have been demonstrated to be strongly associated with increased risk of incident cardiovascular events and mortality in previous follow-up studies.[5,6]

Clinical and laboratory data

Serum lipids, glucose, creatinine, and other biochemical blood measurements were determined in all participants after an overnight fasting using standard laboratory procedures. The estimated glomerular filtration rate (eGFR) was estimated by using the four-variable Modification of Diet in Renal Disease (MDRD) study equation.[18]

Plasma concentrations of high-sensitivity C-reactive protein (hs-CRP), pentraxin-3 (PTX3), and two biomarkers of myocardial damage [high-sensitivity troponin T (hs-TnT) and N-terminal pro-brain natriuretic peptide (NT-proBNP)] were also measured in a single core laboratory.[19]

Body mass index (BMI) was calculated by dividing patients' weight in kilograms by their height in squared metres. Patients were considered to have hypertension if their blood pressure was ≥140/90 mmHg or if they were taking any anti-hypertensive agents. The presence of known diabetes was defined as self-reported physician diagnosis of diabetes or use of any antihyperglycaemic drugs. The LV diameter, wall thickness, and ejection fraction were assessed by trans-thoracic echocardiography according to international standard criteria. More details about clinical and laboratory data have been published elsewhere.[14,15]

Statistical analysis

Owing to the exploratory, hypothesis-generating design of the study, we did not perform *a priori* sample size calculation. Data are expressed as means ± SD when normally distributed, as medians and inter-quartile ranges (IQRs) when non-normally distributed, or as frequencies for categorical variables. The Mann–Whitney *U* test and the Fisher exact test were used to examine differences in clinical and biochemical variables between patients deceased for cardiovascular causes and patients still alive at the end of follow-up. Restricted cubic regression splines were used to formally test the assumption of a linear association between each plasma ceramide [or its ratio with Cer(d18:1/24:0)] and the outcome. Cox proportional hazards regression analysis was used to estimate the hazard ratios (and 95% confidence intervals) associated with the risk of cardiovascular mortality. Associations were presented for every 1-SD increment in plasma ratios of each ceramide with Cer(d18:1/24:0). Four multivariable Cox regression models with pre-specified adjustments were examined: a Model 1 adjusted for age and sex; Model 2 adjusted for age, sex, total cholesterol, and use of any lipid-lowering drugs; Model 3 with further adjustment for heart rate, BMI, NYHA functional class, smoking history, hypertension, diabetes, stroke, atrial fibrillation/flutter, ischaemic HF aetiology, LV ejection fraction, log-transformed serum creatinine, and drug study treatment during the trial (*n*−3 PUFA vs. rosuvastatin); and, finally, Model 4 with additional adjustment for log-transformed plasma PTX3 and NT-proBNP levels. Covariates included in these multivariable Cox regression models were selected based on their significance in univariable analyses or based on their biological plausibility as potential confounding factors. Statistical significance was set at two-sided *P*-value of 0.05. To further correct the analyses for multiple comparisons, we also applied a Bonferroni correction using a significance threshold of 0.01 (0.05/5 plasma ceramide ratios). All statistical analyses were performed using SPSS software (IBM SPSS Statistics for Windows, version 25.0, Armonk, NY) at the Department of

Cardiovascular Research, 'Istituto di Ricerche Farmacologiche Mario Negri IRCCS' of Milan (Italy).

Results

By study design, the cohort comprised a randomly selected sample of 200 ambulatory patients with chronic HF who died due to cardiovascular causes during the follow-up period of the trial (median duration, 3.9 years; IQR, 3.0–4.5 years) and 200 patients who did not (controls). This cohort of 400 patients with chronic HF was representative and well comparable with the respective two cohorts of the GISSI-HF participants who died or did not for cardiovascular causes during the trial (data not shown).

The baseline characteristics of the study participants, stratified by cardiovascular deaths occurred during the trial, are listed in *Table 1*. Compared with survivors, chronic HF

Table 1 Baseline clinical and biochemical characteristics of ambulatory patients with chronic heart failure stratified by cardiovascular deaths at follow-up

	Survivors (*n* = 200)	Deceased (*n* = 200)	*P*-value
Male sex, %	163 (81.5%)	169 (84.5%)	0.505
Age, years	63.8 ± 10.2	70.1 ± 9.5	**6.5 × 10^{-10}**
Age ≥70 years, %	64 (32%)	114 (57%)	**7.2 × 10^{-7}**
NYHA class, III–IV, %	45 (22.5%)	90 (45%)	**5.0 × 10^{-6}**
BMI, kg/m^2	27.1 ± 4.4	26.6 ± 4.3	0.572
BMI ≥ 30 kg/m^2, %	36 (18%)	41 (20.5%)	0.612
Systolic blood pressure, mmHg	131.5 ± 21.5	123.1 ± 18.0	**1.4 × 10^{-4}**
Diastolic blood pressure, mmHg	79.6 ± 10.5	75.3 ± 9.9	**1.2 × 10^{-4}**
Heart rate, b.p.m.	71.4 ± 12.6	75.3 ± 14.1	**0.012**
Diabetes, %	50 (25%)	72 (36%)	**0.022**
Hypertension, %	109 (54.5%)	111 (55.5%)	0.920
Current smokers, %	28 (14%)	14 (7%)	**0.011**
Ischaemic HF aetiology, %	95 (47.5%)	109 (54.5%)	0.193
LV ejection fraction, %	33 [26–39]	31 [25–37]	0.116
LV ejection fraction > 40%, %	26 (13%)	22 (11%)	0.645
Atrial fibrillation/flutter, %	35 (17.5%)	54 (27%)	**0.030**
Pacemaker, %	23 (11.5%)	37 (18.5%)	0.068
Implantable defibrillator, %	16 (8%)	18 (9%)	0.858
Previous myocardial infarction, %	85 (42.5%)	99 (49.5%)	0.192
Angina pectoris, %	25 (12.5%)	37 (18.5%)	0.128
Previous stroke, %	8 (4%)	11 (5.5%)	0.639
Creatinine, mg/dL	1.11 [0.9–1.2]	1.30 [1.0–1.6]	**1.4 × 10^{-8}**
eGFR$_{MDRD}$, mL/min/1.73 m^2	71.2 [56.7–82.8]	57.1 [43.3–70.5]	**2.1 × 10^{-9}**
Haemoglobin, g/dL	14.0 [13.1–15.0]	13.3 [12.2–14.4]	**3.1 × 10^{-5}**
Total cholesterol, mg/dL	200 [171–227]	173 [148–209]	**7.7 × 10^{-7}**
LDL cholesterol, mg/dL	120 [93–141]	100 [75–129]	**2.9 × 10^{-4}**
Triglycerides, mg/dL	130 [89–185]	113 [80–159]	**0.026**
Fasting glucose, mg/dL	103 [92–123]	104 [92–135]	0.432
hs-CRP, mg/L	1.58 [0.78–3.68]	3.15 [1.21–7.51]	**1.0 × 10^{-6}**
PTX3, ng/mL	3.90 [2.83–5.68]	5.05 [3.73–8.26]	**3.0 × 10^{-6}**
hs-TnT, ng/L	15.0 [9.3–21.8]	29.6 [18.9–46.0]	**6.8 × 10^{-21}**
NT-proBNP, pg/mL	699 [314–1245]	2021 [1016–3850]	**1.3 × 10^{-23}**
Concomitant medications			
Open label statins	61 (30.5%)	55 (27.5%)	0.582
ACE-I or ARBs	197 (98.5%)	190 (95%)	0.087
Beta-blockers	133 (66.5%)	115 (57.5%)	0.080
Calcium channel antagonists	17 (8.5%)	16 (8%)	0.998
Aldosterone antagonists	79 (39.5%)	90 (45%)	0.311
Diuretics	182 (91%)	197 (98.5%)	**0.001**
Digitalis	82 (41%)	99 (49.5%)	0.108
Antiplatelets	118 (59%)	99 (49.5%)	0.071
Anticoagulants	55 (27.5%)	84 (42%)	**0.003**
Nitrates	62 (31%)	84 (42%)	**0.029**
Amiodarone	42 (21%)	51 (25.5%)	0.344

Abbreviations: ACE-I, ACE-inhibitors; ARBs, angiotensin II receptor blockers; BMI, body mass index; HF, heart failure; hs-CRP, high-sensitivity C-reactive protein; hs-TNT, high-sensitivity troponin T; LV, left ventricular; eGFR, estimated glomerular filtration rate assessed by the Modification of Diet in Renal Disease study equation; LDL, low-density lipoprotein; NT-proBNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association; PTX3, pentraxin-3.
Cohort size, *n* = 400. Data are presented as mean ± SD, median [IQR], or *n* (%), as appropriate. *P*-value for the Mann–Whitney *U* test or the Fisher exact test, as appropriate. All deaths are due to cardiovascular causes. For the sake of clarity, significant *P*-values are highlighted in bold. Hypertension was defined as blood pressure ≥ 140/90 mmHg or use of any anti-hypertensive drugs. Diabetes was defined as self-reported physician diagnosis of diabetes or use of any antihyperglycaemic agents.

patients who died due to cardiovascular reasons were more likely to be older; were less frequently currently smoking; and had higher heart rate, lower blood pressure, poorer NYHA functional classes, lower eGFR$_{MDRD}$, lower total haemoglobin, lower plasma lipids, and higher prevalence of established diabetes and atrial fibrillation/flutter. These patients also had higher plasma biomarkers of inflammation (i.e. plasma hs-CRP and PTX3) and myocardial damage (i.e. hs-TnT and NT-proBNP). Moreover, they were also more likely to be treated with diuretics, anticoagulants, and nitrates; whereas no significant differences were found in the use of statins, aldosterone antagonists, beta-blockers, calcium channel blockers, digitalis, amiodarone, or anti-platelet agents. Sex, BMI, ischaemic aetiology of HF, LV ejection fraction, and prior history of myocardial infarction, angina, stroke and pacemaker/implantable defibrillator(s) did not significantly differ between the two groups. Drug study treatments were also well balanced between the two patient groups (n−3 PUFAs, 50.5% vs. 47.5%, P = 0.629; rosuvastatin, 28.5% vs. 27.0%, P = 0.911; for survivors and deceased, respectively).

Table 2 shows the baseline levels of plasma ceramides and their individual ratios with Cer(d18:1/24:0) in the two patient groups. Patients who died due to cardiovascular causes had significantly (P < 0.05 or less) higher levels of plasma Cer(d18:1/16:0) and Cer(d18:1/24:1), but lower levels of Cer(d18:1/22:0) and Cer(d18:1/24:0) than those who did not. Additionally, all plasma ratios of each ceramide with Cer(d18:1/24:0) were significantly higher in those who died due to cardiovascular causes than in those who did not.

Figure 1 shows the unadjusted Kaplan–Meier curves for cardiovascular mortality rates among patients stratified by the median value of each ceramide ratio at baseline. Notably, patients who had the plasma ratios of each ceramide with Cer(d18:1/24:0) *above* the median had significantly higher rates of cardiovascular mortality than those with values *below* the median.

In *Figure S1* are reported the restricted cubic spline analyses that we used to formally test the assumption of a linear association between each plasma ceramide [and its ratio with Cer(d18:1/24:0)] and the risk of cardiovascular mortality. Because there was a non-linear association between the individual ceramide species and risk of cardiovascular mortality, a single hazard ratio cannot summarize the association over the range of ceramide values. Thus, for ease of interpretation, we included hazard ratios for the ceramide ratios and restricted cubic spline plots to display the associations between the individual ceramide species and mortality risk.

Table 3 shows the association between plasma ratios of each ceramide with Cer(d18:1/24:0) and the risk of cardiovascular mortality, as well as the effect of the progressive adjustment for multiple established risk factors for HF and potential confounders on these associations in the whole cohort of chronic HF patients. In unadjusted Cox regression analyses, higher plasma ratios of each ceramide with Cer(d18:1/24:0) were significantly associated with higher risk of cardiovascular mortality. Notably, in Cox regression analyses adjusted for multiple known cardiovascular risk factors, serum creatinine, medication use, NYHA functional class, ischaemic HF aetiology, LV ejection fraction, and also drug study treatment during the trial (Adjusted Model 3), we found that all five plasma ceramide ratios maintained a significant positive association with the risk of cardiovascular mortality. In contrast, the additional adjustment for plasma NT-proBNP and PTX3 levels markedly attenuated the significant associations between all plasma ceramide ratios and cardiovascular mortality; in particular, none of these associations remained statistically significant when we also applied a Bonferroni correction for multiple comparisons (Adjusted Model 4).

Table 2 Baseline plasma ceramide levels and their plasma ratios with Cer(d18:1/24:0) in ambulatory patients with chronic heart failure stratified by cardiovascular deaths at follow-up
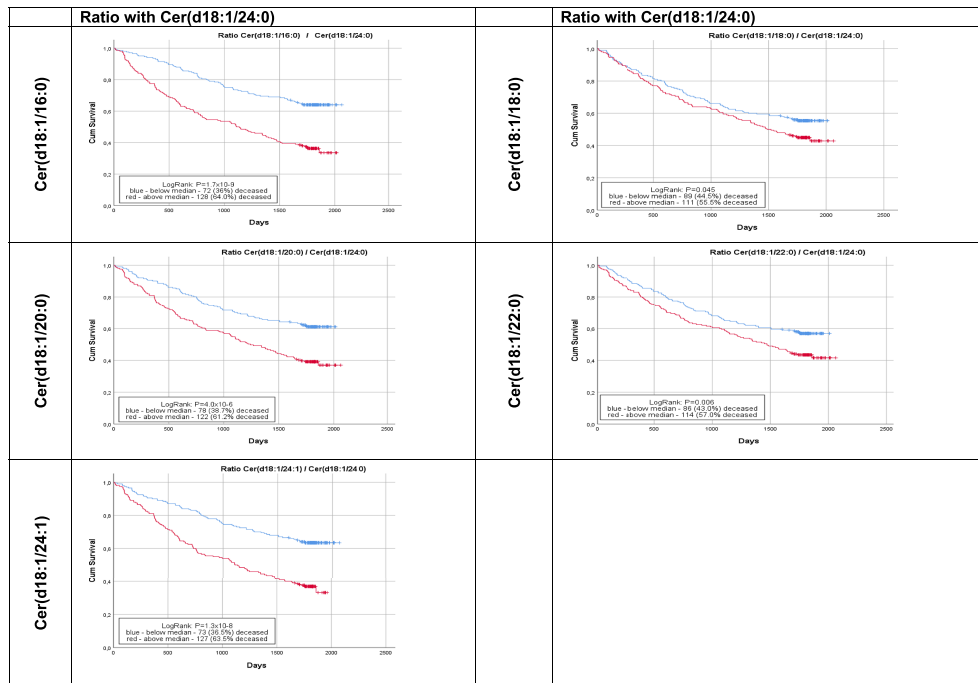
	Survivors (n = 200)	Deceased (n = 200)	P-value[a]
Each plasma ceramide			
Cer(d18:1/16:0), μmol/L	0.179 [0.140–0.221]	0.196 [0.141–0.248]	0.021
Cer(d18:1/18:0), μmol/L	0.094 [0.072–0.119]	0.093 [0.074–0.121]	0.868
Cer(d18:1/20:0), μmol/L	0.080 [0.064–0.095]	0.081 [0.065–0.100]	0.591
Cer(d18:1/22:0), μmol/L	0.529 [0.420–0.633]	0.471 [0.372–0.614]	0.007
Cer(d18:1/24:0), μmol/L	2.269 [1.784–2.622]	1.930 [1.506–2.476]	5.5×10^{-5}
Cer(d18:1/24:1), μmol/L	0.794 [0.634–0.955]	0.828 [0.694–1.021]	0.028
Ratios with Cer(18:1/24:0)			
Cer(d18:1/16:0)/Cer(d18:1/24:0)	0.077 [0.064–0.099]	0.099 [0.074–0.125]	4.4×10^{-9}
Cer(d18:1/18:0)/Cer(d18:1/24:0)	0.042 [0.033–0.055]	0.045 [0.038–0.065]	0.001
Cer(d18:1/20:0)/Cer(d18:1/24:0)	0.036 [0.030–0.042]	0.041 [0.035–0.053]	1.0×10^{-6}
Cer(d18:1/22:0)/Cer(d18:1/24:0)	0.233 [0.210–0.263]	0.244 [0.226–0.272]	0.003
Cer(d18:1/24:1)/Cer(d18:1/24:0)	0.361 [0.292–0.440]	0.447 [0.359–0.568]	6.8×10^{-10}

Abbreviation: Cer, ceramide.
Cohort size, n = 400. Data are presented as median [IQR].
[a]*P*-value for the Mann–Whitney *U* test.

Figure 1 Kaplan–Meier curves for time to cardiovascular mortality in ambulatory patients with chronic heart failure stratified by baseline levels of each plasma ceramide ratio with Cer(18:1/24:0). In these plots, the red line indicates patients with baseline values of each plasma ceramide ratio *above* the median, whereas the blue line indicates those with baseline values of each ceramide *below* the median.

Discussion

The main findings of our hypothesis-generating study are as follows: (i) higher levels of plasma ceramides with long and very-long chains [especially when used in ratios with Cer (d18:1/24:0), which have been demonstrated to be more strongly associated with greater risk of adverse cardiovascular outcomes in previous prospective studies than individual ceramide species[5,6]] were significantly associated with an increased risk of cardiovascular mortality in ambulatory patients with chronic HF; (ii) these significant associations persisted even after adjustment for multiple established risk factors, NYHA functional class, ischaemic HF aetiology, LV ejection fraction, and also drug study treatment during the trial (i.e. $n-3$ PUFA vs. rosuvastatin); and (iii) these associations were substantially weakened after additional adjustment for plasma NT-proBNP and PTX3 levels (especially when we also applied a Bonferroni correction for multiple comparisons).

Recent prospective studies found that higher levels of specific plasma ceramides [especially higher plasma levels of Cer (d18:1/16:0), Cer(d18:1/18:0), and Cer(d18:1/24:1)] were significantly associated with an increased risk of major adverse cardiovascular events both in the general adult population and in cohorts of patients with CAD or acute coronary syndrome, independent of plasma lipids and other known cardiovascular risk factors.[5–9,20] More recently, some

prospective studies have also examined the association between circulating levels of these plasma ceramides and the risk of incident HF, but they provided inconclusive findings.[21,22] Notably, in an individual participant data meta-analysis of the Framingham Heart Study and the Study of Health in Pomerania, Peterson *et al*. found that higher plasma ratios of Cer(d18:1/24:0) or Cer(d18:1/22:0) with Cer(d18:1/16:0) were associated with a significantly lower risk of all-cause and cardiovascular mortality, as well as with a marginally lower risk of incident HF over a mean follow-up of ~6 years.[21] Moreover, in a biracial cohort of 4249 older individuals, Lemaitre *et al*. found that higher circulating levels of Cer(d18:1/16:0) were associated with higher risk of incident HF, whereas higher levels of Cer(d18:1/22:0) were associated with lower risk of incident HF over a median follow-up of ~9.5 years.[22] Plasma levels of Cer(d18:1/20:0) and Cer(d18:1/24:0) were not significantly associated with risk of incident HF.[22]

To our knowledge, this is the first study that has examined the association between plasma ceramides and cardiovascular mortality in a setting of a multicentre randomized controlled trial. Previously, in an observational study involving ~400 elderly Chinese individuals with chronic HF, Yu *et al*. reported that the *total* plasma ceramide concentrations (defined as the *sum* of plasma Cer18, Cer20, Cer22, and Cer24 concentrations) were associated with a higher risk of all-cause mortality, independently of traditional cardiovascular risk factors,

NT-proBNP levels, and LV ejection fraction, over a mean follow-up period of 4.4 years.[23] However, in this observational study, the authors did not perform separate statistical analyses for each distinct plasma ceramide, and no data were available on specific causes of mortality.[23]

To date, the biochemical pathways responsible for greater ceramide synthesis in human HF are not fully understood. Accumulating experimental evidence has suggested that some ceramides, especially those with long and very-long chains, exhibit multiple biological activities that may influence the pathophysiology of both HF and atherosclerotic cardiovascular disease, including effects on cellular apoptosis, oxidative stress, endothelial dysfunction, inflammation, lipotoxicity, insulin resistance, and cardiomyopathy.[1–4,24–26] Experimentally, it has been demonstrated that the myocardium may produce certain ceramides in response to acute ischaemia and reperfusion, leading to an increase of specific ceramides that activate mitochondrial autophagy and apoptosis.[27,28] In addition, Ji et al., analysing the myocardial tissue and serum from patients with advanced HF undergoing placement of LV assist devices, also showed significant increases in long-chain and very long-chain ceramides [mostly Cer(d18:1/16:0) and Cer(d18:1/24:1)] in the failing myocardium and serum of these patients.[29]

Our study has important strengths, such as the prospective design of the study, the inclusion of a relatively large sample of patients with chronic HF belonging to a multicentre randomized controlled trial (i.e. the GISSI-HF, which is essentially a pragmatic all-comer-oriented clinical trial), the use of a 'hard' cardiovascular endpoint that in accordance with the design of the trial was blindly adjudicated in all participants, the completeness of the database with baseline information on many potential confounders, the relatively long duration of the follow-up (median of 3.9 years), and the exclusion of patients with significant liver and kidney diseases. We believe that the inclusion of patients with such co-morbidities might have confounded the interpretation of data.

Among the study limitations, we mention that this is a post-hoc ancillary analysis of the GISSI-HF trial with a case–control design, and we had only a single measurement of plasma ceramides at baseline. Thus, the design of the study does not allow us to establish any causal association between plasma ceramide levels and the risk of cardiovascular mortality. Moreover, the GISSI-HF participants were not necessarily representative of the variety of patients with chronic HF, as the results herein presented pertain to a population of ambulatory patients with chronic HF (mostly with depressed LV ejection fraction), who were followed up by cardiologists and specifically selected to be enrolled in a clinical trial. Given the case–control design of this analysis, it could be difficult to determine whether the associations we observed are specific to cardiovascular mortality or to mortality in general. Additional studies including also chronic HF patients with non-cardiovascular causes of death will be needed to better elucidate this issue. Unfortunately, we did not measure plasma ceramide levels during the

Table 3 Associations between plasma ratios of each ceramide with Cer(d18:1/24:0) and risk of cardiovascular mortality in ambulatory patients with chronic heart failure

Ratios with Cer(18:1/24:0)	Unadjusted model			Adjusted Model 1			Adjusted Model 2			Adjusted Model 3			Adjusted Model 4		
	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Cer(d18:1/16:0)/Cer(d18:1/24:0)	1.56	1.41–1.74	**4.6×10^{-17}**	1.43	1.28–1.60	**3.4×10^{-10}**	1.35	1.20–1.53	**2.0×10^{-6}**	1.30	1.13–1.48	**1.7×10^{-4}**	1.13	0.87–1.47	0.366
Cer(d18:1/18:0)/Cer(d18:1/24:0)	1.37	1.20–1.56	**3.0×10^{-6}**	1.34	1.18–1.54	**1.5×10^{-5}**	1.22	1.06–1.41	**0.006**	1.21	1.04–1.41	0.013	1.10	0.84–1.44	0.486
Cer(d18:1/20:0)/Cer(d18:1/24:0)	1.52	1.34–1.73	**2.6×10^{-10}**	1.46	1.28–1.67	**2.5×10^{-8}**	1.32	1.14–1.54	**1.9×10^{-4}**	1.34	1.13–1.54	**3.9×10^{-4}**	1.34	1.03–1.74	0.031
Cer(d18:1/22:0)/Cer(d18:1/24:0)	1.23	1.08–1.39	**0.001**	1.28	1.12–1.45	**1.9×10^{-4}**	1.16	1.07–1.39	**0.003**	1.22	1.02–1.33	0.028	1.22	0.98–1.52	0.076
Cer(d18:1/24:1)/Cer(d18:1/24:0)	1.59	1.23–1.78	**1.0×10^{-16}**	1.45	1.29–1.63	**7.9×10^{-10}**	1.33	1.15–1.53	**8.0×10^{-5}**	1.30	1.12–1.52	**0.001**	1.34	1.04–1.72	0.024

Cohort size, $n = 400$. Each plasma ceramide (Cer) ratio [expressed as ratio with Cer(18:1/24:0)] has been standardized (for every 1-SD increment). Data are presented as hazard ratio (HR) and 95% confidence interval (95% CI) as assessed by Cox proportional hazards regression analysis. For the sake of clarity, significant P-values under Bonferroni correction (P-threshold 0.05/5 ceramide ratios = 0.01) are highlighted in bold. Adjusted Model 1: adjusted for age (years). Adjusted Model 2: adjusted for age (years), sex (male vs. female), serum total cholesterol (mg/dL) and current use of any lipid-lowering drugs (yes vs. no). Adjusted Model 3: adjusted for age, sex, NYHA functional class (III–IV vs. I–II), heart rate (b.p.m.), BMI (kg/m²), smoking history (yes vs. no), hypertension (yes vs. no), diabetes (yes vs. no), previous stroke (yes vs. no), atrial fibrillation/flutter (yes vs. no), current use of any lipid-lowering drugs (yes vs. no), ischaemic aetiology of HF (yes vs. no), LV ejection fraction (%), serum total cholesterol (mg/dL), log-transformed serum creatinine (mg/dL), and drug study treatments during the trial (i.e. n–3 PUFAs vs. rosuvastatin). Adjusted Model 4: the same covariates included in Model 3 plus log-transformed plasma PTX3 and log-transformed plasma NT-proBNP concentrations.

trial. However, it is reasonable to hypothesize that the two drug study treatments (i.e. $n-3$ PUFA vs. rosuvastatin) did not impact differently on the study outcome(s), because these two drug study treatments were well balanced at baseline between the randomly selected groups of surviving and deceased patients. Finally, we did not measure other biomarkers of the ceramide pathway (e.g. sphingomyelins).

In conclusion, our hypothesis-generating study shows for the first time that higher levels of plasma ceramides with long and very-long chains [especially when used in ratios with Cer (d18:1/24:0)] are significantly associated with an increased risk of cardiovascular mortality, over a median period of 3.9 years, in ambulatory patients with chronic HF. However, these associations are weakened after adjustment for established cardiovascular risk factors, use of medications, and, especially, for plasma NT-proBNP levels (when we also applied a Bonferroni correction for multiple comparisons). Further studies are certainly needed to confirm these results in larger cohorts of ambulatory patients with chronic HF and to investigate pharmacological and nutritional factors that might favourably influence the levels of these plasma ceramides.

Acknowledgements

Conflict of Interest

None.

Funding

Author Contributions

G.T. and L.T. conceived and designed the study. G.T. wrote the draft of the manuscript. G.L., A.M., S.B., P.L.T., E.N., and D.N. analysed the data. J.M. performed the statistical analyses. L.T., A.P.M, and R.L. researched the data, contributed to discussion, and reviewed/edited the manuscript. All authors read and approved the final version of the manuscript. G.T. and R.L. 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.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Restricted cubic spline analyses between each plasma ceramide [and its ratio with Cer(d18:1/24:0)] and risk of cardiovascular mortality. Circulating levels of each ceramide (expressed as either crude values or plasma ratios) are reported on the x-axis, whereas logarithmic hazard ratios for cardiovascular mortality are reported on the y-axis.

Appendix A.

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