Ceramides as Risk Markers for Future Cardiovascular Events and All-Cause Mortality in Long-standing Type 1 Diabetes

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Ceramides are lipid molecules involved in inflammation-related signaling. Recent studies have shown that higher amounts of specific circulating ceramides and their ratios are associated with future development of cardiovascular (CV) disease (CVD). We examined the associations between serum ceramide levels with CVD, kidney failure, and all-cause mortality in individuals with long-standing type 1 diabetes (T1D). We included 662 participants with T1D and 6-year follow-up, with a mean age of 55 years and mean diabetes duration of 33 years. Baseline serum samples were analyzed using liquid chromatography–mass spectrometry. Six predefined ceramide levels were measured, and predefined ratios were calculated. Adjusted Cox regression analyses on ceramide levels in relation to future CV events (CVE), kidney failure, and all-cause mortality were performed, with and without adjustment for age, sex, BMI, LDL, triglycerides, systolic blood pressure, HbA_{1c}, history of CVD, smoking status, statin use, estimated glomerular filtration rate (eGFR), and urinary albumin excretion rate (UAER). The ceramide ratio cer(d18:1/18:0)/cer(d18:1/24:0) was significantly associated with risk of CVE (hazard ratio [HR] = 1.33, *P* = 0.01) and all-cause mortality (HR = 1.48, *P* = 0.01) before and after adjustments. All five investigated ceramide ratios were associated with kidney failure, before adjusting for the kidney markers eGFR and UAER. In this study, we demonstrate specific ceramides and ratios associated with 6-year cardiovascular risk and all-cause mortality in a T1D cohort. This highlights the strength of ceramide association with vascular complications and presents a new potential tool for early risk assessment if validated in other cohorts.

ARTICLE HIGHLIGHTS

- Improved tools for assessing risk for diabetes complication before onset will help in complication prevention.
- We investigated a set of six predefined ceramides and their ratios versus 6-year outcomes of cardiovascular events, kidney failure, and all-cause mortality in people with long-standing type 1 diabetes, using Cox regression with and without adjustment for potential confounders.
- We found that several ceramides and ceramide ratios associated with cardiovascular events and all-cause mortality. The ratio of cer(d18:1/18:0)/cer(d18:1/24:0) was an especially robust marker.
- These finding show that ceramides can be biomarkers of cardiovascular disease and all-cause mortality in individuals with long-standing type 1 diabetes.

The number of people living with type 1 diabetes (T1D) is rising, and estimates foresee further increase in the coming years (1). Two major complications to T1D are cardiovascular (CV) disease (CVD) and chronic kidney disease, the former being the leading cause of death in individuals with T1D (2,3). Individuals with T1D and diabetic kidney disease are subjected to an increased mortality risk (4). Diabetes, CVD, and kidney disease are closely interlinked, sharing risk factors and molecular mechanisms (5,6).

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Identification of biomarkers that stratify those at highest risk for these complications would allow early intervention with optimization of risk factors and potentially organ protective therapies, as has been seen in type 2 diabetes (T2D) with nonsteroidal mineralocorticoid receptor antagonists, glucagon-like peptide 1 receptor agonists (GLP-1RA), and sodium glucose cotransporter 2 inhibitors (SGLT2i) (7,8).

Ceramides are lipid molecules implicated in inflammation and apoptosis signaling (9). Recent studies have found six specific ceramides associated with CV outcomes and mortality years before onset of the clinical disease. Tarasov et al. (10) were among the first to show a link between ceramides and CV risk, when they showed that the amounts of the ceramides cer(d18:1/16:0), cer(d18:1/18:0), cer(d18:1/20:0), and cer(d18:1/24:1), here referred to as cer16, cer18, cer20, and cer24:1, respectively, were significantly higher and cer(d18:1/24:0), referred to as cer24:0, was significantly lower in people who developed coronary artery disease. Tarasov et al. (10) also suggested using the ceramide ratio to cer24:0 to mitigate variations of individual ceramides; these ratios resulted in even stronger association and have been adapted by many subsequent studies. Later studies found cer(d18:1/22:0) (cer22) to associate to CVD an all-cause mortality. Together, these six ceramides and their ratio to cer24:0 have been reported as biomarkers of CV outcomes in multiple large studies (10–16). The levels of these specific ceramides are also increased in individuals with T2D, and have been linked to insulin resistance (17–20) as well as to an increased risk of developing T2D (21–23). However, evidence for ceramide levels as risk predictors for CVD in T1D cohorts is lacking. In this study, we set out to evaluate these six prespecified ceramides in relation to CVD, kidney failure, and all-cause mortality in a prospective study of 662 individuals with T1D.

RESEARCH DESIGN AND METHODS

Participants

This study is based on a prospective cohort study of participants with T1D recruited at the Steno Diabetes Center Copenhagen outpatient clinic between 2009 and 2011, described in full by Theilade et al. (24). A follow-up study was carried out in 2017 obtaining information about hospitalization and death from Danish national registries, as well as carrying out mass spectrometry–based lipidomics analysis using serum samples collected at the start of the original study; details of the follow-up protocol have been reported by Tofte et al. (25). In the current study, a total of 662 participants were included, comprising 296 females (45%) and 366 males (55%).

The original study is registered at ClinicalTrials.gov under the identifier NCT01171248; it holds ethical approval from the Danish National Committee on Biomedical Research Ethics, Copenhagen, Denmark (2009-056). The follow-up protocol was approved The Ethics Committee E, Region Hovedstaden, Hillerød, Denmark. Both the original study and the follow-up protocol adhere to principles of the Declaration of Helsinki. Written and informed consent was given by the participants before inclusion in the original study, which included consent for inclusion in a follow-up study.

Clinical Outcomes

Clinical outcomes at follow-up, followed until 31 December 2016, were assessed using information from the Danish National Death Register and the Danish National Health Register (25–27). Data on hospital admission, diagnoses according to the International Classification of Diseases, Tenth Revision codes, and procedural codes according to the Nordic Classification of Surgical Procedures were obtained from the Danish National Health Register; the specific codes used can be found in Supplementary Table 1. Biochemical measurements including estimated glomerular filtration rate (eGFR) and urinary albumin excretion rate (UAER) were obtained from the local electronic laboratory records.

CV events (CVE) are a composite of several outcomes, as previously defined (28), namely, CV mortality, coronary artery disease including nonfatal myocardial infarction and coronary revascularization (percutaneous arterial intervention or coronary bypass grafting), nonfatal stroke, and peripheral arterial interventions including amputations. For the analyses of any CVE, only the first event was included, even if participants experienced consecutive end points. All deaths, except when an unambiguous non-CV cause of death was reported, were defined as CV mortality. Kidney failure was defined as either receiving dialysis or kidney transplantation or having an eGFR $\leq$15 mL/min/1.73 m^2 (25). Albuminuria status was defined by urinary albumin excretion rate (UAER), normoalbuminuria was defined as having a UAER below 30 mg/g, moderate increase in albuminuria as UAER between 30 and 299 mg/g, and severe increase in albuminuria as UAER $\geq$300 mg/g.

Lipid Analysis

Untargeted lipidomics analysis of baseline serum was carried out using a protocol as previously described in detail (25,29). The analysis was carried out as follows. Serum samples were stored at -80°C and were handled on ice whenever possible. A lipid fraction was created and extracted using a Folch extraction with minor modifications (30). The samples were analyzed using ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry, a detailed description can be found in the Supplementary Material.

The raw mass spectrometry data were analyzed for ceramide amounts as follows: preprocessing was carried out with MZmine 2 v.2.28 (31). In this study, we focused on six prespecified ceramides, namely, cer16, cer18, cer20, cer22, cer24:0, and cer24:1, that were selected based on their consistent association to CV risk as found in the literature. To measure these ceramides, we used their respective water loss adduct $[M\text{-}H2O+H]^+$ as the quantifier ion and the protonated adduct $[M+H]^+$ as a qualifier ion with

matching retention time (32). Final preprocessing was performed in R v.4.2.0 (33). Semiquantification was achieved by comparing ceramide peak areas to the peak area of an exogenous pure standard cer(d18:1/17:0) spiked in all samples for a final concentration of 2 μg/mL. Outliers were defined as measures more than 3 SD away from the median. R code for preprocessing can be found on GitHub: https://github.com/Asger-W/Profil-Ceramides.

Statistics

All statistics and visualizations were produced in R, and the code for the statistical analysis can be found on GitHub. Clinical characteristics presented as *n* (%), mean (SD), or median (interquartile range [IQR]) were compared between individuals developing CVE or those who did not, using Welch *t* test for continuous variables and χ^2 test for categorical variables and compiled into a table with the tableone package (34). Survival analysis was performed with the survival and survminer packages. Cox proportional hazards regression analyses were carried out for each ceramide and ratio to cer24:0, using three levels of adjustments: a crude model without adjustments; a model with level 1 adjustment; and adjusted for age, sex, BMI, LDL, triglycerides, systolic blood pressure, glycated hemoglobin A1c (HbA_{1c}), history of CVD, smoking status, and statin use. The model with final adjustments (level 2 adjustments) included eGFR and log-transformed UAER in addition to the confounders in the level 1 adjusted model. The variables used for adjustment were themselves modeled with Cox regression, in one model without adjustment and in one model with the same adjustments as the level 2 adjusted model except for the interrogated variable. Multiple testing correction was carried out for the six ceramides, with Bonferroni correction *P* value = 0.05/6. Sex was considered as a factor in the statistical analysis of the data, and a separate Cox regression analysis was carried out stratified by sex, for all ceramides, ratios, and outcomes. Correlation matrix of Pearson correlations was produced and drawn as heatmaps with the ggcorrplot package.

Data and Resource Availability

The data set analyzed here is not publicly available, for the privacy of the participants, in compliance with EU and Danish data protection law. The data can be accessed upon request; relevant legal permission from the data protection agency is required. Data access request should be directed to P.R., peter.rossing@regionh.dk. The code used for data analysis is available on gitHub: https://github.com/Asger-W/Profil-Ceramides.

RESULTS

Baseline Characteristics

Here we investigated a prospective cohort of 662 participants with long-standing T1D, baseline lipid measurements, and follow-up data regarding disease and death with median (IQR) follow-up time of 6.3 [5.9–6.7] years. The mean (SD) age was 55 (13) years, the diabetes duration was 33 (16) years, and 45% were female. A total of 94 participants experienced a CVE, 23 progressed to kidney failure, and 58 died; there were 24 people that overlapped between CVE and all-cause mortality, 7 people experienced both a CVE and kidney failure, and 6 people with kidney failure died (Supplementary Fig. 1). Clinical characteristics of the total and subpopulations with and without CVE are summarized in Table 1, and ceramide measures are summarized in Table 2. Ceramide quartiles can be found in Supplementary Table 3. Participants experiencing a CVE had generally higher lipid levels typically associated with CV risk. Total cholesterol, LDL, and VLDL were higher compared with the groups without CVE, but not significantly so, while triglycerides and cer16 and cer18 levels were significantly higher at baseline in the CVE group compared with the non-CVE group (mean [SD] = 2.32 [0.41] ng/mL vs. 2.19 [0.45] ng/mL and 1.36 [0.41] ng/mol vs. 1.24 [0.35] ng/mL, respectively; *P* = 0.007 and 0.008, respectively).

Ceramide Levels and Longitudinal End Points

Cox regression analysis was carried out on the six investigated ceramides to test their association with CVE, kidney failure, and all-cause mortality (Fig. 1).

For CV outcomes, the ratio of cer18/cer24:0 was the only measure that was significantly associated at all levels of adjustments (level 2 adjusted model: hazard ratio [HR] = 1.33, 95% CI = 1.06–1.68, *P* = 0.01). Cer16, cer18, and the ratio cer24:1/cer24:0 were significantly associated with CVE in crude models, but not in adjusted models. Cer22 (HR = 0.72, 95% CI = 0.54–0.97, *P* = 0.03) and the ratio cer16/cer24:0 (HR = 1.32, 95% CI = 1.04–1.67, *P* = 0.02) were statistically significant in the fully adjusted models, but not in the crude model.

In the model with kidney failure as outcome, cer16 and ratios cer20/cer24:0, cer22/cer24:0, and cer24:1/cer24:0 were significantly associated in crude models. In the level 1 adjusted model, all the ratios to cer24:0 were significantly associated with kidney failure (HR = 2.1, 95% CI = 1.37–3.22, $P = 6.56*10^{-4}$), (HR = 1.69, 95% CI = 1.11–2.58, *P* = 0.01), (HR = 1.73, 95% CI = 1.14–2.62, *P* = 0.01), (HR = 1.9, 95% CI = 1.23–2.94, $P = 3.83*10^{-3}$), and (HR = 2.06, 95% CI = 1.38–3.07, $P = 4.14*10^{-4}$) for cer16/cer24:0, cer18/cer24:0, cer20/cer24:0, cer22/cer24:0, and cer24:1/cer24:0, respectively; however, none of these results persisted after inclusion of eGFR and UAER in the model. A full list of model estimates for all the models and adjustment levels can be found in Supplementary Table 4, with a visual summary in Fig. 1.

Cer20, cer22, cer24:0, and cer24:1 were all significantly inversely associated with all-cause mortality, and this finding persisted through all three levels of adjustments. The strongest metabolite associations to all-cause mortality in the adjusted models were cer22 (HR = 0.38, 95% CI = 0.24–0.60, $P = 3.3*10^{-5}$) and cer24:0 (HR = 0.47, 95% CI = 0.31–0.73, $P = 6.6*10^{-4}$). The ratios cer16/cer24:0 and cer18/cer24:0 were also associated with all-cause mortality throughout all

Table 1—Clinical characteristics

	Overall	No CVE	CVE	Groupwise comparison
N	662	568	94	
Age (years)	54.6 (12.7)	53.6 (12.9)	60.7 (9.5)	<0.001
Diabetes duration (years)	32.7 (15.9)	31.3 (15.8)	41.3 (13.1)	<0.001
Female	296 (45%)	264 (47%)	32 (34%)	0.038
Smoking	137 (21%)	116 (20%)	21 (22%)	0.774
BMI (kg/m^2)	25.4 (5.8)	25.4 (6.0)	25.7 (4.2)	0.519
Previous CVD	139 (21%)	85 (15%)	54 (57%)	<0.001
Retinopathy: no/mild-moderate nonproliferative/proliferative	140 (21%)/275 (42%)/ 120 (18%)	133 (23%)/246 (43%)/ 98 (17%)	7 (7%)/29 (31%)/ 22 (23%)	<0.001
UAER (mg/24 h)	17 [8–65]	14 [8–52]	48 [20–219]	<0.001
HbA_{1c} (mmol/mol)	64.3 (12.7)	63.8 (12.6)	67.7 (12.5)	0.006
HbA_{1c} (%)	8.0 (1.2)	8.0 (1.2)	8.3 (1.1)	0.006
Hemoglobin (mmol/L)	8.5 (0.9)	8.5 (0.8)	8.2 (1.0)	0.012
Total cholesterol (mmol/L)	4.7 (0.9)	4.7 (0.8)	4.8 (1.0)	0.193
HDL (mmol/L)	1.7 (0.5)	1.7 (0.5)	1.6 (0.6)	0.089
LDL (mmol/L)	2.5 (0.8)	2.4 (0.7)	2.6 (0.9)	0.138
VLDL (mmol/L)	0.5 (0.3)	0.5 (0.3)	0.6 (0.3)	0.03
Triglyceride (mmol/L)	1.13 (0.66)	1.09 (0.58)	1.35 (1.01)	0.015
eGFR (mL/min/1.73m^2)	81.53 (25.51)	83.95 (24.69)	67.00 (25.68)	<0.001
hsCRP (mg/L)	3.41 (7.02)	3.36 (7.20)	3.70 (5.83)	0.616
Systolic blood pressure (mmHg)	132 (17)	131 (17)	137 (19)	0.004
Diastolic blood pressure (mmHg)	74 (9)	75 (9)	73 (10)	0.089
Below albuminuria/moderately increased/severely increased (<30/30–299/≥300 mg/g)	308 (47%)/165 (25%)/ 189 (29%)	290 (51%)/133 (23%)/ 145 (26%)	18 (19%)/32 (34%)/ 44 (47%)	<0.001
RAAS treatment*	445 (67%)	358 (63%)	87 (93%)	<0.001
Antihypertensive treatment	475 (72%)	382 (67%)	93 (99%)	<0.001
Beta blocker treatment	85 (13%)	58 (10%)	27 (29%)	<0.001
Calcium channel blocker treatment	202 (31%)	155 (27%)	47 (50%)	<0.001
Insulin pump user	57 (9%)	52 (9%)	5 (5%)	0.303
Daily insulin dose (IU)	48.7 (35.0)	48.8 (36.2)	47.9 (26.3)	0.781
Statin treatment	397 (60%)	321 (57%)	76 (81%)	<0.001
Aspirin or clopidogrel treatment	349 (53%)	273 (48%)	76 (81%)	<0.001
Diuretic treatment	334 (51%)	260 (46%)	74 (79%)	<0.001
Thiazide treatment	186 (28%)	159 (28%)	27 (29%)	0.982
Loop diuretic treatment	149 (23%)	105 (19%)	44 (47%)	<0.001
MRA treatment	28 (4%)	17 (3%)	11 (12%)	<0.001

Data are presented as *n* (%), mean (SD), or median [IQR]. Groupwise comparisons between the two treatments were tested using a Welch two-sample *t* test for continuous variables and χ^2 test for categorical variables. MRA, mineralocorticoid receptor antagonist; RAAS, Renin angiotensin aldosterone system. *Spironolactone that blocks aldosterone is given separately.

levels of adjustments(HR = 1.69, 95% CI = 1.27–2.23, $P = 2.8*10^{-4}$ and HR = 1.48, 95% CI = 1.12–1.94, $P = 5*10^{-3}$, respectively, in the fully adjusted model).

The ratio of cer18/cer24:0 was the only measure that was significantly associated with CVE and all-cause mortality at all levels of adjustments. Kaplan-Meier curves (Fig. 2) show that participants above the median of ratio of cer18/cer24:0 were at higher risk (81% survival rate, 95% CI = 77–85, 6 years after measuring) than participants below the median (89% survival rate, 95% CI = 85–92, 6 years after measuring,

Table 2—Ceramide measures

	Overall	Individuals without CVE	Individuals who developed CVE	Groupwise comparison
n	662	568	94	
Cer16 ng/mL	2.21 (0.44)	2.19 (0.45)	2.32 (0.41)	0.007*
Cer18 ng/mL	1.26 (0.36)	1.24 (0.35)	1.36 (0.41)	0.008*
Cer20 ng/mL	1.78 (0.66)	1.78 (0.66)	1.83 (0.71)	0.523
Cer22 ng/mL	10.26 (4.28)	10.24 (4.22)	10.43 (4.65)	0.707
Cer24:0 ng/mL	39.99 (16.29)	39.94 (16.00)	40.30 (18.06)	0.855
Cer24:1 ng/mL	19.55 (7.44)	19.32 (7.20)	20.93 (8.66)	0.09
Ratio cer16/cer24:0	0.06 (0.02)	0.06 (0.02)	0.07 (0.03)	0.137
Ratio cer18/cer24:0	0.03 (0.01)	0.03 (0.01)	0.04 (0.01)	0.019
Ratio cer20/cer24:0	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)	0.261
Ratio cer22/cer24:0	0.26 (0.04)	0.26 (0.04)	0.26 (0.04)	0.461
Ratio cer24:1/cer24:0	0.51 (0.11)	0.50 (0.11)	0.54 (0.12)	0.005*

Data are presented as mean (SD). Groupwise comparisons between the two groups were tested using a Welch two-sample *t* test. *Indicate passing Bonferroni correction.

P = 0.013) for CVE. The ceramide ratio cer18/cer24:0 was better at differentiating CVE from non-CVE than LDL, based on which participants above the median of LDL were at a similar risk (84% survival rate, 95% CI = 0.80–0.88, 6 years after measuring) to the participants with LDL lower than the median (87% survival rate, 95% CI = 0.83–0.91, 6 years after measuring, P = 0.2).

Examining the variables used for adjustment, we found that CVD history was the variable with the strongest association with CVE (HR = 3.72, 95% CI = 2.34–5.92, $P = 2.84*10^{-8}$), Supplementary Fig. 2 and Supplementary Table 5. The ceramides showed strongest correlation with each other and with the other lipid measures like LDL and triglycerides correlation coefficients between 0.3 and 45 and between 0.27 and 0.5, respectively, Fig. 3, with extended heatmap in Supplementary Fig. 3. In addition, cer24:0 had a small positive correlation with eGFR (0.08), compared with the other ceramides that had negative or no correlation with eGFR.

We investigated how the HRs for CVE were affected by albuminuria and found that, from normoalbuminuria to moderate increase in albuminuria, cer16 and cer18 were significantly associated with CVE, but, in the subpopulation with severely increased albuminuria, all significant associations were lost (Fig. 4). The levels of cer18 and cer24:0 and the ratio of cer18/cer24:0 were plotted for each albuminuria group and for CVE (Supplementary Fig. 4 and Supplementary Table 6), which shows that the difference in ceramide level between individuals with and without a CVE is gradually smaller with worsening albuminuria.

Finally, sensitivity analysis was carried out, investigating the effect of antihypertensive treatment, antiplatelet drugs, Renin-angiotensin-aldosterone system (RAAS) treatment, and HDL; adjusting for these variables had little or no influence on the models and is therefore not included. A Cox regression analysis carried out in females and males separately (Supplementary Fig. 5) showed that the ceramides and the ceramide ratios associated to CVE in males but not in females. All the ceramide ratios associated to kidney failure in males, while, in females, only cer22, cer24:1, and ratio cer22/24:0 associated to kidney failure. The same ceramide and ceramide ratios associate to all-cause mortality in both sexes, but the associations were a bit stronger in males. We also found that the association between ceramides and kidney failure was stronger in people who had not previously had a CVD; similarly, the association between ceramides and all-cause mortality was strongest in people without previous CVD (Supplementary Fig. 6). A *t* test of ceramide amount, comparing people with and without previous CVD, showed that ratios cer18/cer24:0 and cer18/cer24:0 was significantly higher in people with previous CVD than in those without (Supplementary Table 7).

DISCUSSION

We investigated the potential of a small set of specific circulating ceramides as biomarkers for CV risk, kidney failure, and all-cause mortality in individuals with T1D. In brief, the key findings are as follows. *1*) Several of the investigated ceramides associated with CVE risk and all-cause mortality in individuals with T1D. *2*) The cer18/cer24:0 ratio was the most consistent measure and showed constant, significant association with CVE and all-cause mortality across all confounder adjustment levels. The cer18/cer24:0 ratio furthermore outperformed LDL cholesterol in separating individuals who progressed to a CVE from the group who did not. *3*) Ratios of the ceramide cer24:0 associated, albeit more weakly, to kidney failure, until the adjustment for UAER and eGFR. The loss of association after adjustment to UAER and eGFR may be explained by collinearity between ceramide ratios and eGFR (Fig. 3). *4*) We observed that the ceramides' ability to differentiate between

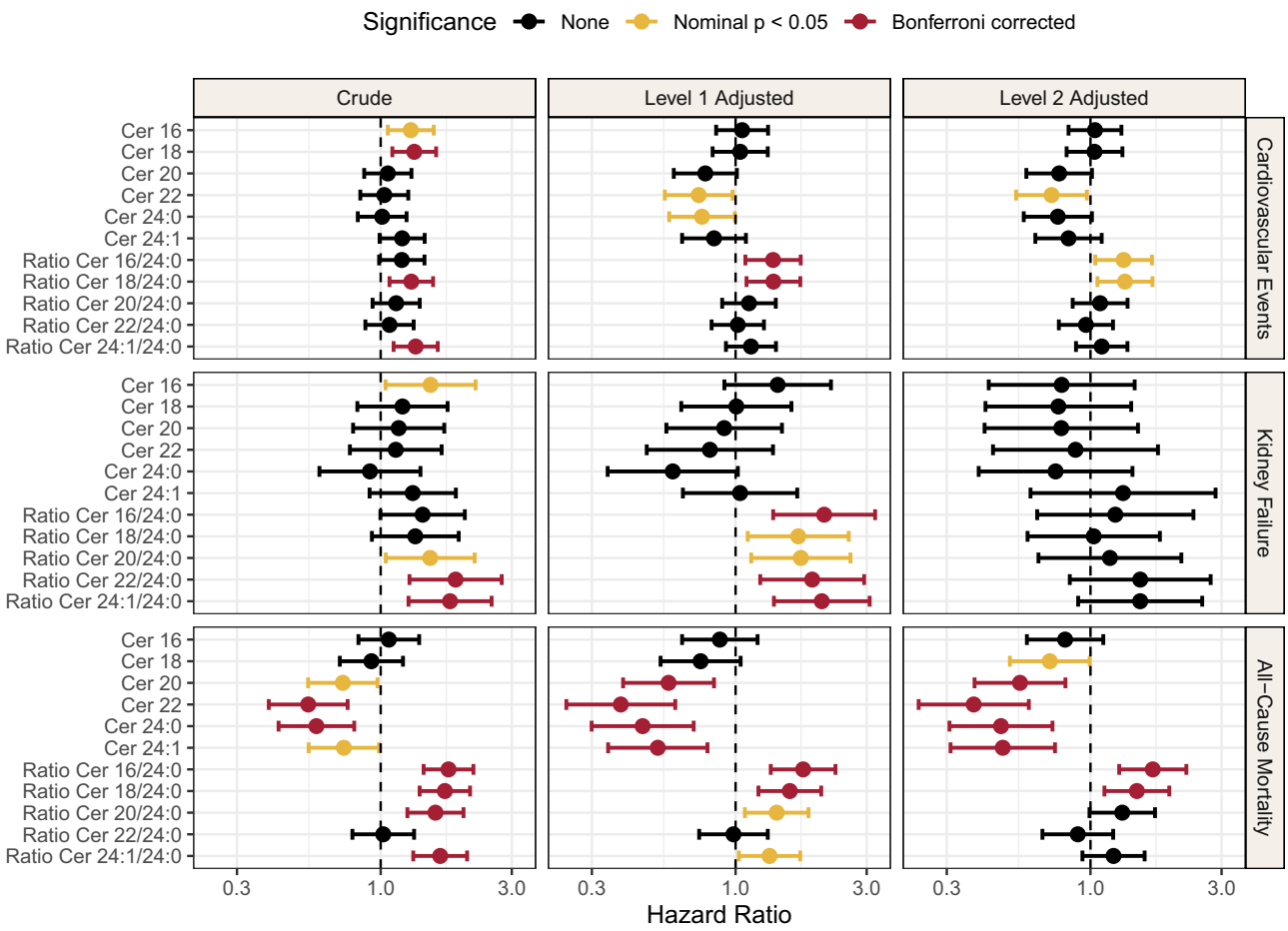

Figure 1—Forest plot of HRs for ceramide and ratios for outcomes of CVE, kidney failure, and mortality. The crude models are unadjusted; level 1 adjusted for age, sex, BMI, LDL, triglycerides, systolic blood pressure, HbA_{1c}, history of CVD, smoking status and statin use; and level 2 adjusted for all the same variables as level 1, but also including eGFR and UAER. HRs are reported per doubling of the log10 ceramide.

individuals with and without future CVE depended on albuminuria status; whether this is a direct effect or an indicator of compounding illness is not clear, though it should be noted that these ceramides have previously been associated with albuminuria and eGFR (35–37). Interestingly, the ceramides are not correlated with traditional risk factors such as BMI or blood pressure (Supplementary Fig. 3). Previous studies found that including ceramides measures improved upon the ability of traditional lipid measurements like LDL and TG to predict major adverse cardiac events and suggested ceramides associate to CVD independently of established clinical risk factors (12,38).

There is compelling evidence that ceramides can predict CVD risk. Tarasov et al. (10) were among the first to report increased levels of cer16, cer18, cer20, and cer24:1 as risk markers of coronary artery disease, together with a reduced risk associated with cer24:0. Several subsequent studies found associations for ceramide levels and CV risk (39–41) and severity (42); however, few have studied these biomarkers in relation to diabetes. Alshehry et al. (43) investigated lipids associated with CVE and CV death in a T2D cohort and found that, among other lipids, cer24:1 was positively associated with CVE and CV death. We did not observe an association between cer24:1 and CVE; however, we did observe a protective association to all-cause mortality. We have not been able to find any studies investigating the CV risk of these ceramides in people with T1D. Lipid measures are well-established measures of CVD risk (44,45). We found that the ratio of cer18/cer24:0 could be a comparable measure to LDL. Havulinna et al. (12) showed that adding cer18 measures to LDL levels improved their ability to predict incident rate of CVE, while adding LDL levels to cer18 level did not improve prediction. Several mechanisms have been suggested for the observed link between ceramides and CVD. One plausible explanation is that ceramides are recruited during inflammation to induce apoptosis in dysregulated cells such as foam cells (46). It has been suggested that ceramides have a causative role in promoting CVD (47), for instance, through mitochondria disruption, promoting reactive oxygen species and cell death (48–50).

The association between ceramides and kidney disease in the literature is less clear. One study in T1D found that

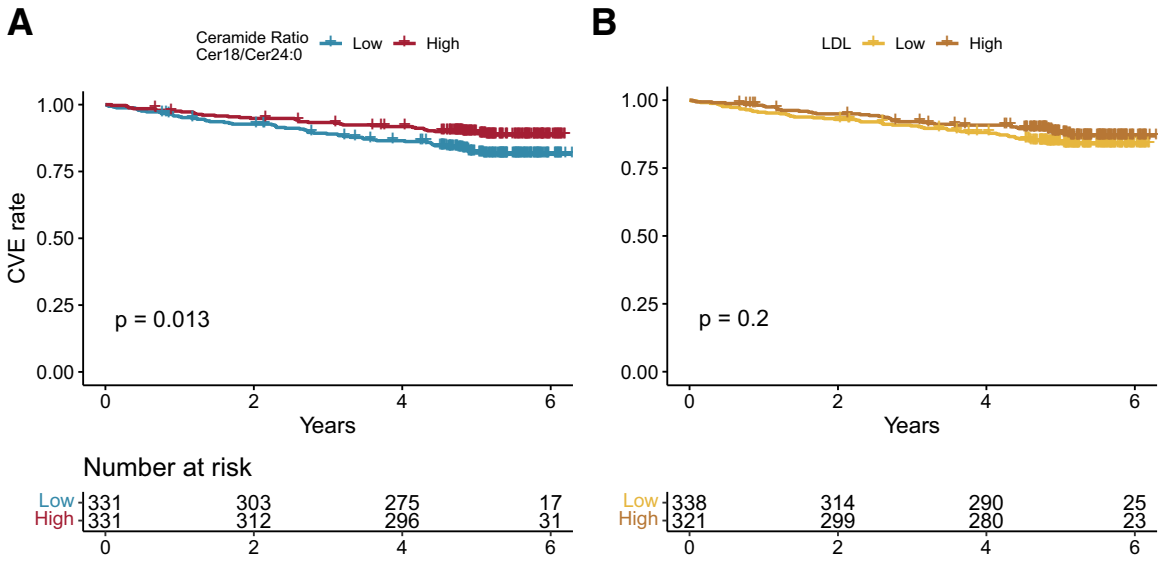

Figure 2—Kaplan-Meier plot of (*A*) cer18/cer24:0 ratio and (*B*) LDL against CVE. High is metabolite level greater than or equal to the median; low is individuals with a metabolite level below the median.

individuals with lower ceramide levels of cer20 and cer24:1 at baseline were more likely to progress to macroalbuminuria, with an average 6.5-years follow-up (35). The people included in that study were both younger, with a mean age of 27 years, and had a shorter diabetes duration, 6 years on average, compared with our study, where the participants were middle aged, a mean age of 55 years, and had long-standing T1D, 33 years on average, which could explain why we do not find the same associations for cer20 and cer24:1. On the other hand, other studies have found an increase in ceramide levels in people with chronic kidney disease (37) and diabetic kidney disease (36) compared with controls without kidney disease. The increase of cer16 found in these two studies is consistent with the association between cer16 and risk of kidney failure found in this study (Fig. 1). The relationship between ceramides and kidney disease is complex and likely bidirectional. Elevated ceramide levels could contribute to the development and progression of kidney disease, while kidney dysfunction may also contribute to changes in ceramide metabolism. Considering these reports and our study, it is possible that ceramide levels increase with kidney/endothelial damage up until a tipping point, where the levels then drop; a similar metabolic tipping point has been suggested in individuals with severe liver fibrosis (51). The influence of albuminuria status on ceramides' ability to associate with CVE should be investigated further and be considered when using these ceramides as risk predictors.

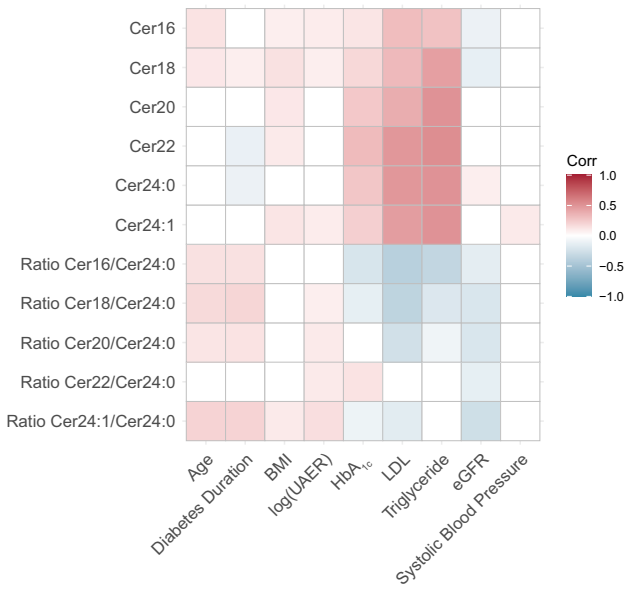

Figure 3—Heatmap of ceramides correlation to possible CVE confounders, presented as correlations coefficients from Pearson correlation.

This study has some limitations. We were not able to control for diet, exercise, and change in medication in this post hoc analysis, which could have influenced ceramide levels (15,19). It should be noted that the participants had long-standing T1D and therefore were likely to adhere to a low-carb diet without major changes. Information on race was not available but was expected to represent the Danish population. Another limitation was that we did not have information on the use of medication targeting triglycerides, which potentially could affect ceramide levels. Despite the possible variability introduced by uncontrolled factors, we found robust associations between ceramides, CVE, and all-cause mortality. Validating our finding in an independent replication cohort would help cement our results further; however, we were not able to identify a similar cohort. For the strengths, the levels of the ceramides were measured in a semiquantitative manner allowing for comparison with other

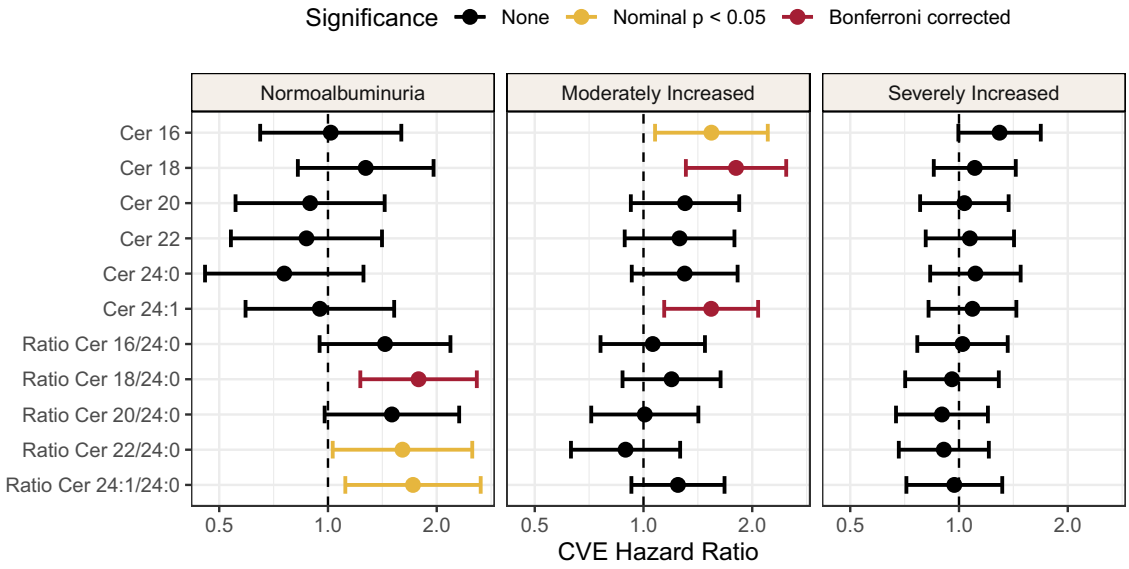

Figure 4—Forest plot of HRs for ceramide and ratios for CVE separated by albuminuria status. Normoalbuminuria is defined as <30 mg/g, moderately increased is between 30 and 299 mg/g, and severely increased is ≥300 mg/g. These models are unadjusted crude models. HRs are reported per doubling of the log10 ceramide.

future studies. Additionally, investigating few specific molecular targets measured in concentrations reduces the risk of incidental discoveries. It was also a strength that this analysis was carried out in a large, well-characterized prospective cohort.

In this study, we investigated a small set of specific ceramides in relation to CVE, kidney failure, and all-cause mortality. We found that these ceramides have potential as susceptibility biomarkers of CVD and all-cause mortality in individuals with long-standing T1D. The ratio cer18/cer24:0 appears to be the strongest independent risk marker for CVD and all-cause mortality, and future work should focus on validating it in clinical practice.

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Author Contributions. A.W. performed data analysis and drafted the manuscript. A.W., V.R.C., T.S., S.T., N.T., S.A.W., T.V., H.V., P.R., and C.L.-Q. contributed to the conceptualization and interpretation of this study. S.T. and P.R. conducted the original study. V.R.C., T.S., N.T., and S.A.W. carried out the follow-up study and provided material and clinical data for this study. All authors approved the final version of the manuscript. A.W. and C.L.-Q. 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 analysis.

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