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Ceramides and Phosphatidylcholines Associate with Cardiovascular Diseases in the Elderly

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BACKGROUND: The ceramide- and phospholipid-based cardiovascular risk score (CERT2) has been found to predict the risk for cardiovascular disease (CVD) events, especially cardiovascular mortality. In the present study, our aim was to estimate the predictive ability of CERT2 for mortality of CVD, coronary artery disease (CAD), and stroke in the elderly and to compare these results with those of conventional lipids.

METHODS: We conducted a prospective study with an 18-year follow-up period that included a total of 1260 participants ages ≥64 years. Ceramides and phosphatidylcholines were analyzed using a LC-MS. Total cholesterol and triglycerides were performed by enzymatic methods and HDL cholesterol was determined by a direct enzymatic method. Concentrations of LDL-cholesterol were calculated according to the Friedewald formula.

RESULTS: A higher score of CERT2 was significantly associated with higher CVD, CAD, and stroke mortality during the 18-year follow-up both in unadjusted and adjusted Cox regression models. The unadjusted hazard ratios (HRs) of CERT2 (95% CI) per SD for CVD, CAD, and stroke were 1.72 (1.52–1.96), 1.76 (1.52–2.04), and 1.63 (1.27–2.10), respectively, and the corresponding adjusted HRs (95% CI) per SD for CERT2 were 1.48 (1.29–1.69), 1.50 (1.28–1.75), and 1.41 (1.09–1.83). For conventional lipids, HRs per SD were lower than for CERT2.

CONCLUSIONS: The risk score CERT2 associated strongly with CVD, CAD, and stroke mortality in the elderly, while the association between these events and conventional lipids was weak.

Introduction

The prevalence and associated mortality of cardiovascular diseases (CVD) is a major clinical challenge. The identification of patients at highest risk for cardiovascular (CV) events is essential for individualized treatment. The risk of CV events in patients with CVD varies substantially between individuals (1–5). For the past few decades, conventional lipid concentrations have been used commonly to assess the risk of CV events (6). With increasing age, the importance of previously studied CV risk predictors (e.g., different lipoprotein subclasses) seems to change (7–12). Whereas most studies have been performed in middle-age populations, there are variable results on the role of classic risk factors in different age groups (13), and only a few longitudinal studies in this regard have been published (14).

Ceramides belong to the class of sphingolipids. They consist of a sphingosine backbone and a fatty acid chain whereas phosphatidylcholines (PCs) have a choline head group and two fatty acyl side chains. Ceramides are essential components of cell membranes of eukaryotic cells (15). They are also signal effector molecules and involved in regulation of several biologically important functions like cell differentiation, cell stress, inflammatory signaling, and regulation of cell death. In earlier studies, ceramides have been associated with obesity and insulin resistance (16–18). Blood ceramides

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and their distinct ratios have been found to predict CV mortality and acute cardiac events better than conventional lipids in patients with stable coronary artery disease (CAD) and in apparently healthy populations (19–22). The best predictive risk score for CV mortality has been obtained using a test based on ceramides and phospholipids (21, 23). Hilvo et al. published data derived from 3 large clinical cohorts in which patients were age 30 to 75 years (24). Another study confirmed these results in a large SOLID-TIMI-Study (25). The ceramide- and phospholipid-based cardiovascular risk score (CERT2) consists of a ceramide/ceramide ratio, 2 ceramide/PC ratios, and a single PC. The ceramide-PC ratio components of the CERT2 test showed higher hazard ratios than the previously published ceramide–ceramide ratios in CERT1 (19, 21, 23).

With populations aging worldwide, the number of CAD patients continues to increase. The aim of this study was to estimate the CERT2 in the elderly population. The main interest was to assess whether the CERT2 analysis offers an advantage over conventional lipids in predicting CVD, CAD, and stroke mortality in a population of elderly people.

Material and Methods

STUDY DESIGN AND POPULATION

The study population was from the Lieto Elderly Study, a longitudinal epidemiological study conducted in southwestern Finland in the municipality of Lieto. All inhabitants born in 1933 or earlier (age ≥ 64 years) were invited to participate in the baseline study ($n = 1596$). Of those who were eligible, 63 died before they were examined, and 273 refused to participate or did not respond. A total of 1260 (82%) persons (533 men and 727 women) participated in the study. Clinical examinations were conducted between March 1998 and September 1999. At baseline, the study protocol consisted of an extensive interview, clinical examination, and numerous laboratory tests (26). The population was followed up for 18 years for CVD, CAD, and stroke mortality.

LABORATORY MEASUREMENTS

Conventional lipid analyses were performed using fresh samples. Blood samples were collected, centrifuged at 2100 g for 10 min, and aliquots of serum (1 mL cryotube) were stored at −70°C. Ceramides were analyzed from stored samples that had not been previously thawed. The stability of ceramide and phospholipids decreased with sample storage at room temperature without extraction. The stability of lipids is shown in the online Supplemental Table S1–S2. Due to the limited volume of some samples, lipid and ceramides analyses could be performed in only 1119 individuals of the original Lieto study population.

Total cholesterol and triglycerides were determined by an enzymatic method, and HDL cholesterol analysis was performed by a direct enzymatic method (Roche Diagnostics and Hitachi 917 analyzer). Concentrations of LDL-cholesterol were calculated using the Friedewald formula (27).

To evaluate CERT2, extraction and analysis were performed as follows: Extraction: 10 µL of sample matrix was transferred to a 2 mL 96 well plate and 590 µL of isopropanol:ethyl acetate (4:1 v:v) containing deuterated internal standard molecules for each lipid was added to the wells. The plate was mixed for 5 min and then centrifuged 10 min at 3000 g. 50 µL of the organic phase was transferred to a 96 well plate for LC-MS/MS analysis. All standards and deuterated internal standards were purchased from Sigma Aldrich (manufacturer Avanti polar lipids, catalog numbers listed in Table 1). Phosphatidylcholine 14:0/22:6 and 16:0/22:5 were synthesized on request using Avanti polar lipids, as were their deuterated internal standards.

Quantitative LC-MS/MS analysis was conducted on a Sciex TripleQuad 5500 mass spectrometer coupled to a Sciex MPX LC system. Electrospray ionization in positive ion mode was performed with multiple reaction monitoring. Instrument and data acquisition were monitored using Analyst® (version 1.7). The following settings were applied to all compounds in the analysis: curtain gas, 35; ion spray voltage, 5000 V; temperature, 300°C; gas 1 and gas 2, 50; declustering potential, 30; entrance potential, 10; collision exit potential, 20. Collision energy was set separately to each lipid. An Acquity BEH C18 2.1 × 75 mm id. 1.7 µm column was used for chromatographic separation. The temperature was set to 60°C. Mobile phases consisted of (A) 10 mmol/L ammonium acetate with 0.1% formic acid and (B) 10 mmol/L ammonium acetate in acetonitrile:2-propanol (4:3, v:v) with 0.1% formic acid. Loading pump solvent in the MPX consisted of A:B (21:79%). Injection volume was 3 µl and flow rate was 500 µl/min. The following gradient was applied: 80% B from 0 to 0.5 min, then to 84% B at 1 min and to 89% B at 2.4 min. Solvent B was set to 100% at 2.5 min and held at 100% to 4.5 min, then dropped to 80% at 4.6 min and held at 80% until 5 min. Both streams had the same parameters. Mass spectrometry analysis was performed from 1.8 min to 4.4 min, which allowed multiplex to run a sample every 2.5 min. Each 96 well plate had a standard line (6 points), 6 QC samples, and blank samples to ensure analytical quality through the whole sample range. Measured *m/z* values and collision energies are reported in Table 1. The QC data and chromatograms of CERT2 are shown in the online Supplemental Table S3 and Fig. S1. The analytical

Table 1. Measured *m/z* values and collision energies for all the measured lipids and their internal standards.

Ceramides	Q1 (M + HCOO)- (*m/z*)	Q3 (M + HCOO)- (*m/z*)	Collision energy	Catalog number
18:1/16:0	538.5	264.3	40	860516P
18:1/16:0—d7	545.5	271.3	40	860676P
18:1/18:0	566.5	264.3	40	860518P
18:1/18:0—d7	573.5	271.3	40	860677P
18:1/24:0	650.6	264.3	40	860524P
18:1/24:0—d7	657.6	271.3	40	860678P
18:1/24:1	648.6	264.3	40	860525P
18:1/24:1—d7	655.6	271.3	40	860679P
Phosphatidylcholines				
14:0/22:6[a]	778.5	184.1	40	NA
14:0/22:6—d9	787.5	193.1	40	NA
16:0/16:0	734.6	184.1	15	850355P
16:0/16:0—d9	743.6	193.1	15	860352P
16:0/22:5[a]	808.6	184.1	15	NA
16:0/22:5—d9	817.6	193.1	15	NA

[a][2-[(4Z,7Z,10Z,13Z,16Z)-docosa-4,7,10,13,16-pentaenoyl]oxy-3-hexadecanoyloxypropyl] 2-(trimethylazaniumyl)ethyl phosphate.
Abbreviations: NA, not applicable.

method was validated according to the Food and Drug Administration guideline for biological sample analyses.

OUTCOME MEASURES

Unique personal identification numbers of all participants who died before January 2017 were used to obtain data from the official Finnish Cause of Death Registry. Deaths coded according to the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (28) codes I21, I22, I23, I24, I25, and I46 were classified as CAD deaths and codes I61, I63 (excluding code I63.6), and I64 as stroke deaths (28). Deaths resulting from CAD or stroke were combined as CVD deaths.

ETHICS

The Lieto Elderly Study was conducted according to the guidelines of the Declaration of Helsinki. The Ethics Committee of the Hospital District of Southwest Finland approved the study protocol (2018). Participants provided written informed consent for the study.

STATISTICAL ANALYSES

The calculation of the CERT2 score has been described previously (22, 25). In brief, 4 components, that is, the Cer(d18:1/16:0)/PC(16:0/22:5), Cer(d18:1/18:0)/PC(14:0/22:6), and Cer(d18:1/24:1)/Cer(d18:1/24:0) lipid ratios, as well as PC(16:0/16:0) concentration, were used to calculate the score (scale 0–12 points). The calculation of the CERT2 are shown in the online Supplemental Method for Calculating the CERT2 Risk Score.

Baseline characteristics of the cohorts were described using medians (interquartile range) as well as minimum and maximum for continuous variables and numbers (percentages) for categorical variables. Uni- and multivariate Cox proportional hazard regression models were used to determine hazard ratios (HRs) and 95% CI for the associations of CERT2 with incident events. The models were stratified by sex, and in multivariable models the adjustments were made for logarithmic body mass index, logarithmic age, smoking, diabetes, and hypertension, and the effects for body mass index and age were expressed per SD. The risk curves are for all the events during the entire follow-up period. They were constructed with the *ggplot2* package using the loess method, which is a nonparametric univariate regression method. All tests were 2-sided and $P < 0.05$ was considered as statistically significant. R version 4.0.2 was used for all statistical analyses.

Results

BASELINE CHARACTERISTICS

The baseline characteristics of 1119 study participants are presented in Table 2. The mean age of the participants was 72.7 years, and 41% were male.

Table 2 Baseline characteristics of the participants in the Lieto Elderly Study ($n = 1119$).

Characteristics	n (%)
Male	464 (41)
Diabetes	129 (12)
Hypertension	409 (37)
Prevalent CVD	229 (20)
	Median (IQR), min–max
Age, years	72.7 (68.7–78.2), 64.7–100.0
BMI, kg/m^2 (n = 1116)	26.8 (24.2–29.8), 14.2–48.9
CERT2 (n = 1119)	6 (4–8), 0–12
Total cholesterol, mg/dL (n = 1118)	220 (193–247), 81–406
HDL cholesterol, mg/dL (n = 1118)	54 (46–66), 23–124
LDL cholesterol, mg/dL (n = 1105)	139 (116–162), 35–298
Triglycerides, mg/dL (n = 1118)	115 (89–159), 35–770

Abbreviations: IQR, interquartile range.
To convert total cholesterol, HDL cholesterol, LDL cholesterol concentrations to mmol/L, multiply by 0.0259.
To convert triglyceride concentrations to mmol/L, multiply by 0.0113.

Regarding performance of CERT2 and conventional lipids in risk assessment, the quality of the CERT2 measurements was confirmed using 6 quality control samples in each 96 well plates. Calculated relative SDs in percentage were <9.1% for all lipids through the entire study. Specific values can be found in the online Supplemental Table S3.

The performance of CERT2 and conventional lipids were evaluated for CVD, CAD, and stroke mortality. For CVD, CAD, and stroke mortality, the unadjusted HRs (95% CI) per SD for CERT2, respectively, were 1.72 (1.52–1.96), 1.76 (1.52–2.04), and 1.63 (1.27–2.10), and the corresponding adjusted HRs (95% CI) per SD for CERT2 were 1.48 (1.29–1.69), 1.50 (1.28–1.75), and 1.41 (1.09–1.83). For conventional lipids, HRs per SD were lower than for CERT2. A higher score of CERT2 was significantly associated with higher CVD, CAD, and stroke mortality during the 18-year follow-up both in unadjusted (in the online Supplemental Table S4) and adjusted Cox regression models (Table 3). No significant associations were found between conventional lipids and CVD, CAD, or stroke mortality in adjusted models.

CERT2 risk curves for fatal CVD, fatal CAD, and fatal stroke in the Lieto elderly cohort population are shown in Fig. 1, A–C. As the score increased, the risk appeared to increase consistently. The associations between CERT2 risk groups and clinical outcomes were presented using Kaplan–Meier curves. Survival probability of fatal CVD with 4 different CERT2 score categories in this elderly cohort population are demonstrated in Fig. 2A. Survival probabilities of CAD are shown in the online Supplemental Figure S2A. The data showed that, with increasing CERT2 score, fatal events increased during the 18-year follow-up period. For comparison, Kaplan–Meier curves for HDL cholesterol, LDL cholesterol, total cholesterol, and triglycerides are shown for CVD in Fig. 2, B–E, and for CAD in the online Supplemental Figure S2, B–E. The Kaplan–Meier curves showed that there was no clear separation among the 4 groups for conventional lipids.

Discussion

Cardiovascular diseases are the most common cause of mortality in Western countries. They start to develop in early childhood and progress depending on genetic, environmental, nutritional, and other lifestyle factors. With age, the spectrum of diseases, risk factors, morbidity, and mortality changes. However, knowledge of the impact of various CV risk factors at different ages is based primarily on cross-sectional studies or the studies that have been performed in middle-age populations. There are also contradictory results concerning the role of lipids and their subfractions as risk factors for the elderly in comparison to their role in younger people (14).

As a result, ceramides have been intensively investigated as new risk factors for CVD to replace or supplement conventional lipid markers. Several studies indicate that a combination of distinct ceramides and validated score like CERT1 will be superior to conventional lipid measurements. In this study, we applied CERT2 instead

Table 3 Adjusted HRs and their 95% CI of CERT2 and conventional lipids for CVD, CAD, and stroke mortality during the 18-year follow-up among the population of the Lieto Elderly Study.

	CVD mortality		CAD mortality		Stroke mortality	
	Number of deceased 254		Number of deceased 189		Number of deceased 65	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
CERT2	1.48 (1.29–1.69)	<0.001	1.50 (1.28–1.75)	<0.001	1.41 (1.09–1.83)	0.010
Total cholesterol	1.12 (0.98–1.29)	0.088	1.14 (0.97–1.33)	0.110	1.10 (0.85–1.42)	0.470
HDL cholesterol	0.95 (0.82–1.10)	0.480	0.98 (0.83–1.15)	0.780	0.87 (0.65–1.16)	0.350
LDL cholesterol	1.15 (1.01–1.31)	0.041	1.15 (0.98–1.34)	0.080	1.14 (0.90–1.46)	0.280
Triglycerides	1.04 (0.91–1.19)	0.580	1.04 (0.89–1.21)	0.640	1.05 (0.79–1.40)	0.720

The models were stratified by sex, and in multivariable models the adjustments were made for logarithmic BMI, logarithmic age, smoking, diabetes, and hypertension, and the effects for BMI and age were expressed per SD.

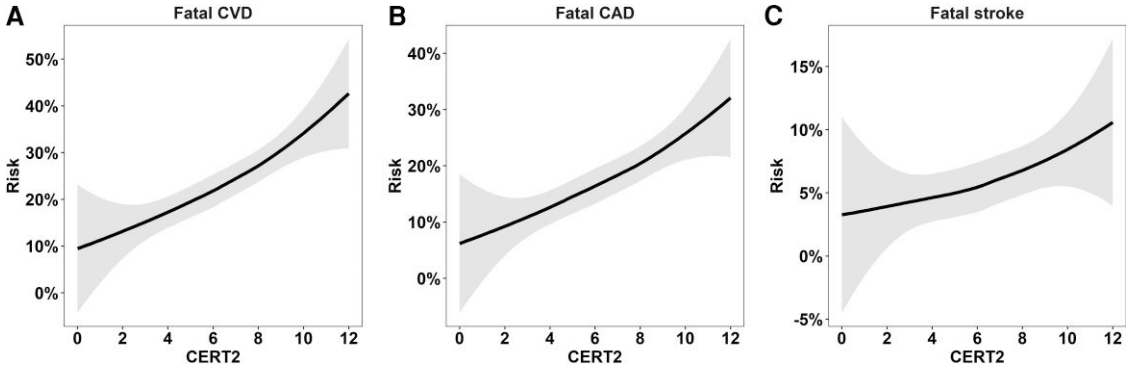

Fig. 1. Risk curves for CERT2 for (A) fatal CVD, (B) fatal CAD, and (C) fatal stroke during the 18-year follow-up among the population of the Lieto Elderly Study.

of CERT1 for risk evaluation (19, 21, 23, 29–32). In addition, the use of statins has not been specifically addressed in this study, as their use among those over 64 years of age was relatively low at the time of sample collection in 1998–99. Statin treatment was analyzed by Hilvo et al. and did not show a significant interaction in the LIPID study (24).

The current study was one of the first to explore the CERT2 score as a risk indicator in the older population. We showed that the risk score CERT2 is significantly associated with CVD, CAD, and stroke mortality. In addition, CERT2 associates with the aforementioned events more highly than conventional lipids. These results are to be expected, as it has been shown that conventional lipids have a rather poor predictive value in the aging population. The FINRISK 2002, which is a population-based risk factor survey, has shown, for example, that the predictive value of LDL-cholesterol is poor in middle age and older (>50 years) individuals (12). Limitations of this study were the relatively small sample material, the retrospective nature of the study, and long storage times of the samples.

Ceramides and PCs were analyzed in a research laboratory, and other lipid analyses were performed in the clinical laboratory. Currently, ceramide analysis needs a liquid chromatography-mass spectrometer and related expertise. Thus, ceramide analytics have been considered difficult to use in routine clinical laboratories (33). On the other hand, in specialized departments of clinical laboratories, many analytes, such as hormones, are determined by mass spectrometer. Thus, a standardized and validated analytical method as described in the present paper is possible to set up on existing mass spectrometers in a majority of the reference laboratories (34).

Ceramide analysis appears to be an effective way to assess the risk of CVD, CAD, and stroke mortality in elderly. The critical question is what the essential risk assessment means for this age group (≥64 years).

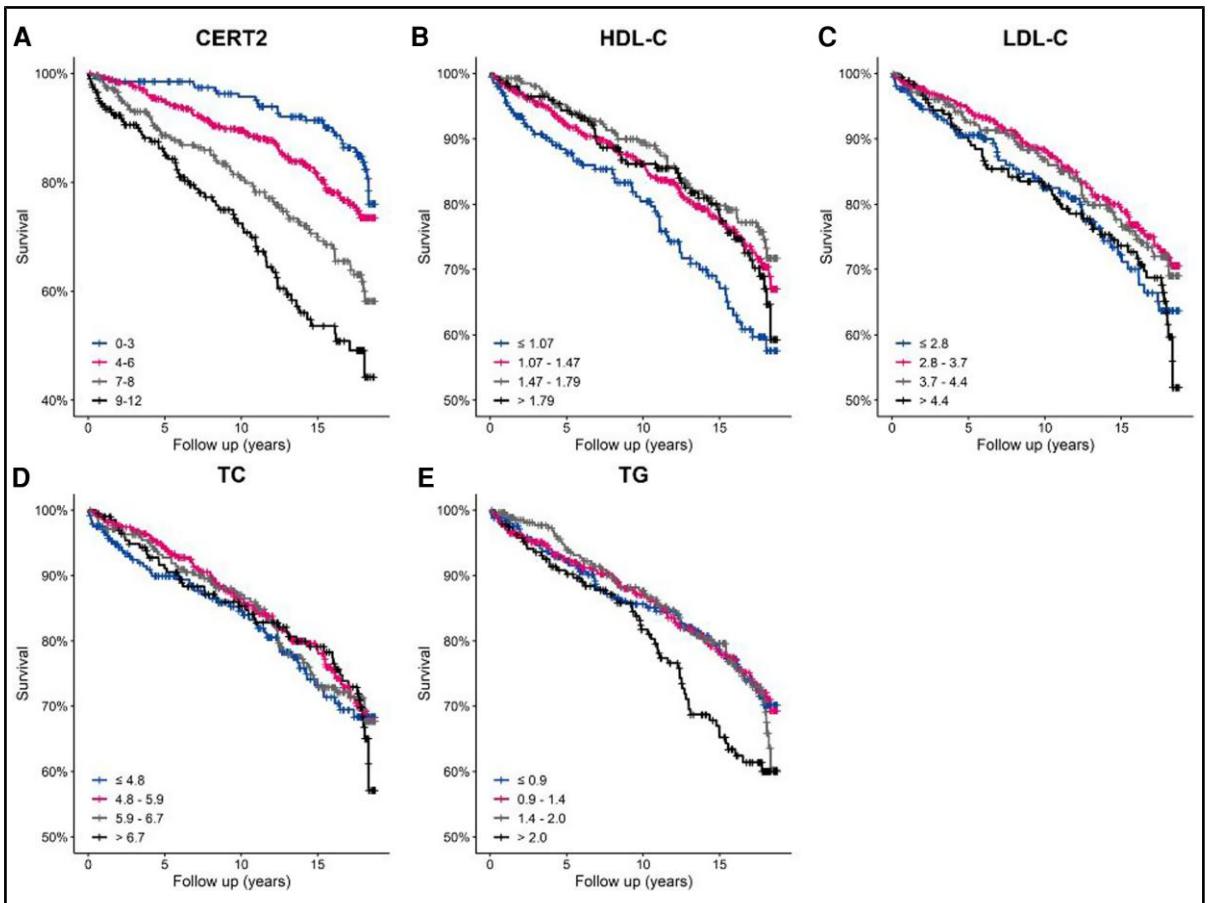

Fig. 2. A Kaplan-Meier for (A) CERT2 and conventional lipids, (B) HDL chloresterol, (C) LDL chloresterol, (D) total cholesterol (TC), (E) triglycerides for CVD during the 18-year follow-up among the population of the Lieto Elderly Study.

Improved risk stratification may aid in decision-making related to the medical treatment strategy and patient follow-up. On the other hand, a person over the age of 64 identified as high risk could further change his or her lifestyle (e.g., exercise, diet) or influence treatment compliance, leading to an increase in healthy life years and thus a potential reduction in healthcare costs. More precise risk estimation may also aid in avoiding unjustified drug treatments in the elderly, which may reduce risk of drug interactions and related adverse events.

Supplementary Data

Supplementary data is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: CVD, cardiovascular disease; CV, cardiovascular; PC, phosphatidylcholine; CAD, coronary artery disease; CERT2, ceramide- and phospholipid-based cardiovascular risk score; HR, hazard ratio.

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