# Circulating Ceramides Predict Cardiovascular Outcomes in the Population-Based FINRISK 2002 Cohort

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***Objective***—Ceramides are molecular lipids implicated in apoptosis, inflammation, obesity, and insulin resistance. An earlier study reported that ceramides were associated with fatal outcome among patients with coronary heart disease. Here, we examined whether ceramides are associated with major adverse cardiovascular events (MACEs) among apparently healthy individuals.

***Approach and Results***—FINRISK 2002 is a population-based risk factor survey, which recruited men and women aged 25 to 74 years. The cohort was followed up until the end of 2014. We quantified 4 circulating ceramides, Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:0), and Cer(d18:1/24:1), in 8101 serum samples by a targeted LC-MS/MS assay. Primary outcome of interest was incident MACE (n=813). Secondary analyses were performed for MACE death (n=116) without previous nonfatal MACE and for recurrent MACE (n=226) among survivors of a previous incident MACE. We used Cox proportional hazard models adjusted for the Framingham covariates to determine the association of ceramides with the outcomes. Of the ceramide species, Cer(d18:1/18:0) had the strongest association with incident MACE and the highest unadjusted hazard ratio of 1.31 (95% confidence interval, 1.21–1.41), which remained significant at 1.21 (95% confidence interval, 1.11–1.33) after Framingham risk factor adjustments. The hazard ratios were generally stronger for recurrent and fatal events than for first events. Clinical net reclassification improvement was 7.5% ($P$=6.9×10$^{-5}$) for Cer(d18:1/18:0).

***Conclusions***—Distinct serum ceramides are associated with the risk of incident MACE in apparently healthy individuals. These results should encourage more detailed analyses of ceramides in cardiovascular pathobiology and suggest new biomarkers of MACE risk. (***Arterioscler Thromb Vasc Biol*. 2016;36:00-00. DOI: 10.1161/ATVBAHA.116.307497.)**

**Key Words:** cardiovascular diseases ■ ceramides ■ lipids ■ metabolomics ■ risk factors





Serum lipids, including total cholesterol, low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C), have all been used in risk prediction of atherosclerotic heart disease, and LDL-C has been established as the main lipid target in the management of patients with coronary artery disease (CAD). On the basis of broad lipidomic analyses in different CAD patient cohorts, we and others have reported that other circulating lipid species may also hold prognostic potential in CAD.[1–4]

In our earlier studies, ceramide molecules Cer(d18:1/16:0), Cer(d18:1/18:0), and Cer(d18:1/24:1), as well as their ratios to Cer(d18:1/24:0), have emerged as novel risk stratifiers in patients with established CAD. These ceramides are analytically robust and have been proven to provide significant incremental value over routinely used lipid markers in a secondary prevention setting, particularly for hard outcomes such as cardiovascular death.[5] Literature on ceramide biology associates these molecules with many central atherosclerotic processes, such as lipoprotein aggregation, uptake of lipoproteins and accumulation of cholesterol within macrophages, regulation of nitric oxide synthesis, production of superoxide anions, and expression of different cytokines.[6–11] Furthermore, experimental studies have shown that ceramides and related sphingolipids are associated with the development of atherosclerosis.[6] Inhibition of glycosphingolipid biosynthesis decreased atherosclerosis in mice, and consequently, several enzymes of the sphingolipid synthetic pathway have been tested as potential drug targets.[12,13]

As an extension of observations in secondary prevention cohorts, the present study evaluated ceramide behavior in the large-scale prospective FINRISK study to validate the prognostic value of the ceramides at the population level.

## Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

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| Nonstandard Abbreviations and Acronyms | |
|---|---|
| **CAD** | coronary artery disease |
| **HDL-C** | high-density lipoprotein-cholesterol |
| **LDL-C** | low-density lipoprotein-cholesterol |
| **MACE** | major adverse cardiovascular event |
| **NRI** | net reclassification index |

## Results

### Elevated Ceramide Concentrations Are Associated With Increased Major Adverse Cardiovascular Event Risk

This study included 8101 individuals from the FINRISK 2002 general population cohort. In short, median age was 48.5 years, and 53% of the study subjects were women. A preexisting atherosclerotic vascular disease was present in 3.6%, whereas 5.5% had diabetes mellitus and 25.7% of the subjects were smokers at study baseline. The baseline characteristics of the cohort are summarized in Table I in the online-only Data Supplement. During a follow-up of 13 years, 813 subjects experienced an incident major adverse cardiovascular event (MACE), of which 116 were fatal (annotated as MACE death throughout this work). Table 1 shows serum concentrations of TC, LDL-C, HDL-C, TG, ceramides, and CRP for asymptomatic subjects and for subjects who experienced an incident MACE during the follow-up. Increased concentrations of distinct ceramides were observed in individuals who experienced an incident MACE during the follow-up compared with asymptomatic individuals. The serum concentrations of the 3 culprit high-risk species that were previously shown to predict cardiovascular death in CAD patients were also higher in FINRISK MACE cases compared with asymptomatic subjects as follows: Cer(d18:1/16:0), Cer(d18:1/18:0), and Cer(d18:1/24:1) 11.4%, 21.3%, and 17.0% ($P$<0.001 for all), respectively. Two predefined ceramide ratios, Cer(d18:1/18:0)/Cer(d18:1/24:0) and Cer(d18:1/24:1)/Cer(d18:1/24:0), were also significantly higher in subjects with an incident MACE compared with asymptomatic subjects.

### Ceramide (d18:1/18:0) Associates With Incident MACE

The association between ceramides and MACE was further evaluated by the Cox regression analyses. Figure 1 shows the associations of the ceramides with incident MACE, including fatal and nonfatal MACE, and separately for MACE death only.

The 3 high-risk ceramides were associated with incident MACE and MACE death. The Cer(d18:1/18:0) displayed the highest univariate hazard ratio (HR) for incident MACE (1.31; 95% confidence interval, 1.21–1.41), which remained significant (1.21; 95% confidence interval, 1.11–1.33) after adjusting for the Framingham Risk Score factors. Also, the highest univariate HR for MACE death (1.46; 95% confidence interval, 1.20–1.78) was observed for Cer(d18:1/18:0), but it did not remain significant after adjustment for the Framingham Risk Score factors. Subjects with a known prevalent MACE were excluded from these analyses.

### Ceramide (d18:1/18:0) Improves Clinical NRI in the FINRISK 2002 Population

The incremental prognostic value of ceramides was tested by comparing the Framingham score base model with a new model adding ceramides on top of the Framingham score. The predicted probabilities for a 10-year risk of incident MACE by cross-validated Weibull regression yielded significant clinical net reclassification index (NRI) for the 3 high-risk ceramide species (Table II in the online-only Data Supplement). In line with the above-mentioned association results, Cer(d18:1/18:0) showed the largest clinical NRI (7.5%; $P$=6.9×10$^{-5}$), which was mainly driven by down-classification (6.9%; $P$=1.2×10$^{-5}$) of risk among people

**Table 1. Medians and Interquartile Ranges of Established Lipid Markers and Ceramides in Participants Without MACE During the Follow-Up and With Incident MACE**

| | MACE$^-$ (n=6892) | MACE$^+$ (n=813) | Significance |
|---|---|---|---|
| Cer(d18:1/16:0) | 0.28 (0.24–0.33) | 0.33 (0.28–0.38) | *** |
| Cer(d18:1/18:0) | 0.11 (0.09–0.15) | 0.15 (0.11–0.19) | *** |
| Cer(d18:1/24:0) | 2.28 (1.86–2.77) | 2.60 (2.14–3.14) | *** |
| Cer(d18:1/24:1) | 1.52 (1.25–1.85) | 1.77 (1.47–2.19) | *** |
| Cer(d18:1/16:0)/Cer(d18:1/24:0) | 0.13 (0.11–0.14) | 0.13 (0.11–0.14) | |
| Cer(d18:1/18:0)/Cer(d18:1/24:0) | 0.05 (0.04–0.06) | 0.06 (0.05–0.07) | *** |
| Cer(d18:1/24:1)/Cer(d18:1/24:0) | 0.67 (0.59–0.76) | 0.70 (0.61–0.79) | *** |
| LDL-C | 3.30 (2.72–3.90) | 3.70 (3.09–4.34) | *** |
| HDL-C | 1.46 (1.21–1.75) | 1.28 (1.12–1.57) | |
| TC | 5.48 (4.85–6.22) | 5.94 (5.21–6.62) | *** |
| TG | 1.16 (0.84–1.66) | 1.50 (1.16–2.18) | ** |
| CRP | 1.10 (0.51–2.44) | 1.94 (0.95–4.63) | * |

CRP indicates XXX; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; MACE, major adverse cardiovascular event; TC, XXX; and TG, XXX.

The Wilcoxon test ***$P$<0.001; **$P$<0.01 and *$P$<0.05.

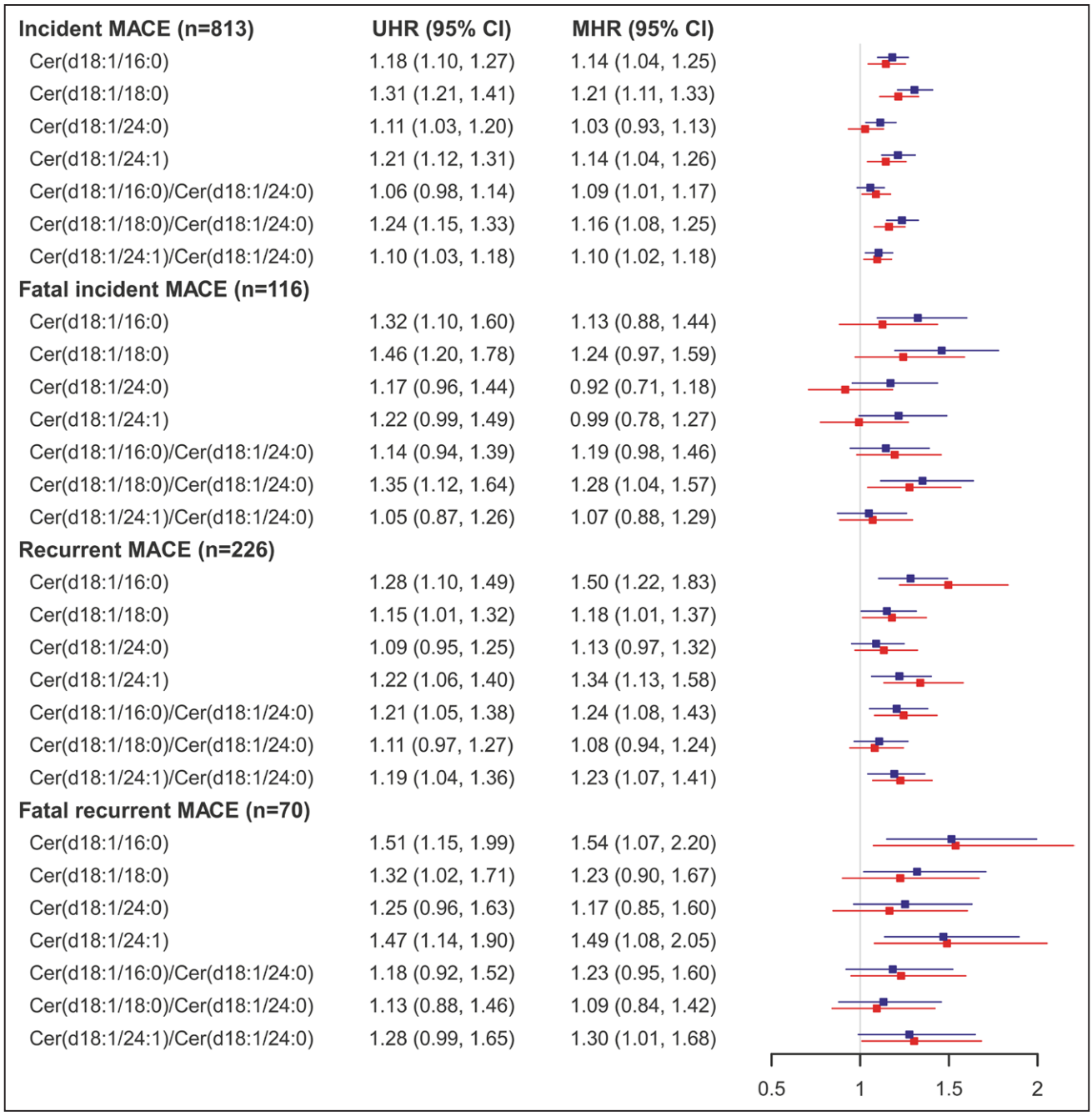


**Figure 1. Upper**, Unadjusted (UHR, blue) and adjusted (MHR, red) hazard ratios for incident major adverse cardiovascular event (MACE) and fatal incident MACE for participants with no MACE before baseline (n=7705). Adjustment for Framingham risk factors: total cholesterol, high-density lipoprotein-cholesterol (HDL-C), blood pressure (adjusted +15 mm Hg for antihypertensive medication), diabetes mellitus, and smoking. **Lower**, Unadjusted (UHR, blue) and adjusted (MHR, red) hazard ratios for recurrent MACE and fatal recurrent MACE for patients with prevalent MACE at baseline (n=396). Adjustment for Framingham risk factors: total cholesterol, HDL-C, blood pressure (adjusted +15 mm Hg for blood pressure medication), diabetes mellitus, and smoking. Age was used as the time scale in all models, and the models were stratified by sex. Therefore, the unadjusted models are essentially equivalent for age- and sex-adjusted models.

in the intermediate risk group (5%–20% 10-year MACE risk). However, C-index as a discrimination measure remained nonsignificant, as well as the NRI for the fatal end points.

### Ceramide (d18:1/18:0) Adds Value to Current Lipid and CRP Measurements

The significant associations between incident MACE and distinct ceramides prompted us to test whether ceramides could add prognostic value to the currently used routine clinical laboratory measures, such as CRP, HDL-C, or LDL-C. Correlation analysis showed weak to moderate correlations of measured ceramides and their ratios with CRP and routinely used lipids (Table III in the online-only Data Supplement). Of note are the relatively strong correlations of Cer(d18:1/18:0) with CRP and lipids, except for HDL-C. However, despite the observed correlation between ceramides and CRP, the

association between ceramides and MACE remained practically unchanged after additional adjustment for CRP on top of the Framingham risk score variables (Figure I in the online-only Data Supplement) compared with adjustment for the Framingham variables only (Figure 1).

We selected Cer(d18:1/18:0) for more detailed analyses because it showed the strongest association with incident MACE in the analyses summarized above. To assess the potential synergy between currently used clinical assays and Cer(d18:1/18:0), we divided the samples into concentration quartiles by CRP and Cer(d18:1/18:0), by HDL-C and Cer(d18:1/18:0), and by LDL-C and Cer(d18:1/18:0), and calculated the incident MACE rate percentages in these overlapping quartiles across the combinations (Figure 2).

Figure 2A shows that the lowest 13-year incident MACE risk (2.6%) was observed in subjects having both CRP and Cer(d18:1/18:0) in the lowest concentration quartile (first quartile [Q1]) and the highest risk (20.9%) was found in subjects having both CRP and Cer(d18:1/18:0) in the highest concentration quartile (fourth quartile [Q4]). High >15% risk was observed in subjects having CRP concentrations >0.52 mg/dL (Q2–Q4) and Cer(d18:1/18:0) in Q4. The synergy of the CRP and Cer(d18:1/18:0) combination was further evidenced by the Kaplan–Meier estimates of incident MACE showing the high risk in subjects with top quartile serum concentrations for both CRP and Cer(d18:1/18:0) (Figure 3).

**A**

| CRP \ Cer(d18:1/18:0) | Q1 | Q2 | Q3 | Q4 |
|---|---|---|---|---|
| 0.05-0.52 | 2.6 | 5.2 | 7.7 | 7.0 |
| 0.53-1.13 | 4.9 | 8.6 | 13.4 | 15.7 |
| 1.14-2.53 | 6.7 | 8.7 | 11.9 | 16.2 |
| 2.54-132.8 | 10.1 | 11.9 | 14.9 | 20.9 |

**B**

| HDL-C \ Cer(d18:1/18:0) | Q1 | Q2 | Q3 | Q4 |
|---|---|---|---|---|
| 0.31-1.20 | 8.3 | 13.2 | 17.3 | 18.4 |
| 1.21-1.45 | 6.8 | 7.6 | 10.6 | 16.9 |
| 1.46-1.74 | 3.2 | 6.2 | 11.5 | 15.1 |
| 1.75-3.77 | 3.5 | 7.1 | 9.5 | 15.9 |

**C**

| LDL-C \ Cer(d18:1/18:0) | Q1 | Q2 | Q3 | Q4 |
|---|---|---|---|---|
| 0.77-2.71 | 3.6 | 7.0 | 11.3 | 16.7 |
| 2.72-3.29 | 5.8 | 7.4 | 12.5 | 16.8 |
| 3.30-3.89 | 6.9 | 8.7 | 10.9 | 17.3 |
| 3.90-9.55 | 8.1 | 11.0 | 14.1 | 16.6 |

**Figure 2. A–C**, Incident major adverse cardiovascular event (MACE) rate (%) in different concentration quartiles of Cer(d18:1/18:0), CRP, low-density lipoprotein-cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL-C).

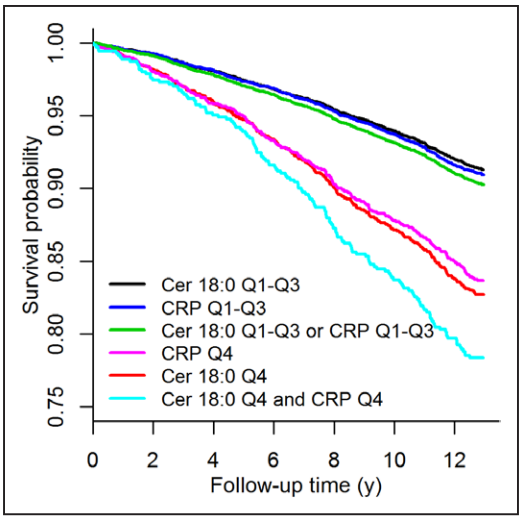


**Figure 3.** Kaplan–Meier estimates of incident major adverse cardiovascular event (MACE) for subpopulations with Cer(d18:1/18:0) in Q4 vs not in Q4, CRP in Q4 vs not in Q4, and Cer(d18:1/18:0) in Q4 and CRP in Q4 vs its complement.

HDL-C seemed to perform similarly as Cer(d18:1/18:0), but when combined, more cases mapped to the higher concentration Cer(d18:1/18:0) (Q4) and lower concentration HDL-C (Q1) quartiles where the MACE risk was 18.4% (Figure 2B). When mapped together with LDL-C, the case enrichment was mainly evident because of the effects of higher concentrations of Cer(d18:1/18:0) across Q4 (Figure 2C). These data are further illustrated in Figure 4 separately for Cer(d18:1/18:0) and LDL-C by Kaplan–Meier curves for incident MACE per concentration quartile showing a steeper slope and higher HRs for Cer(d18:1/18:0).

## Ceramide (d18:1/16:0) and (d18:1/24:1) Associate With Recurrent MACE

Our previous studies indicate that ceramides serve as prognostic markers for fatal outcome in patients with CAD.[5,14] With this in mind, we separately analyzed subjects who already had experienced MACE before the baseline measurement (n=396) and as the end point those who had a recurrent MACE (n=226) or fatal recurrent MACE (n=70) during follow-up. For recurrent MACE, Cer(d18:1/16:0) and Cer(d18:1/24:1) showed significant univariate associations, with Cer(d18:1/16:0) having the highest univariate HR at 1.28 (1.10–1.49), which remained significant at HR=1.50 (1.22–1.83) also after adjusting for the Framingham Risk Score factors (Figure 1).

For those patients who had experienced MACE before baseline measurement, the association with MACE death was significant for Cer(d18:1/16:0) and Cer(d18:1/24:1). The univariate HR was highest at 1.51 (1.15–1.99) for Cer(d18:1/16:0), and it remained significant at HR=1.54 (1.07–2.20) after adjusting for the Framingham Risk Score factors.

## Ceramide Score Associates With MACE at Population Level

Because the data above show that the occurrence of incident MACE on one hand, and recurrent MACE on the other hand,

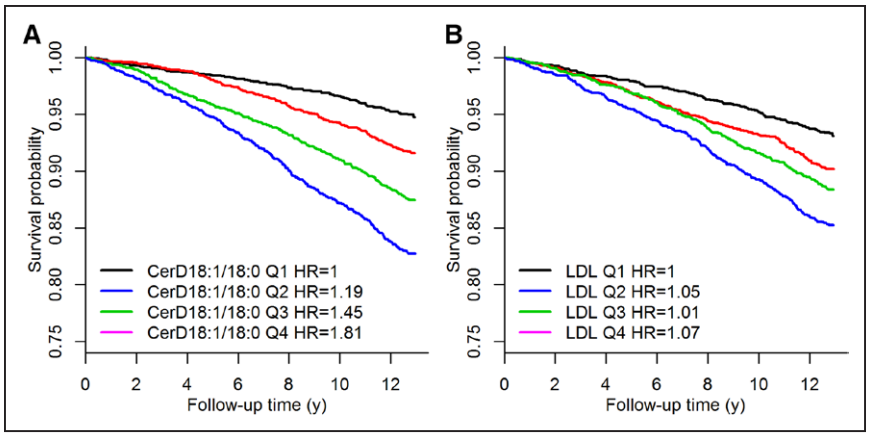


**Figure 4. A** and **B**, Kaplan–Meier estimates of incident major adverse cardiovascular event (MACE) Cer(d18:1/18:0) and low-density lipoprotein-cholesterol (LDL-C) quartiles. A hazard ratio in the legend indicates the quartile-specific hazard relative to the first quartile (models stratified for sex).

may be associated with different ceramides, we applied a previously developed ceramide risk score[14] to evaluate the association between ceramides and MACE in the whole study population. On the basis of this score, the patients were placed into 4 risk categories (low, moderate, increased, and high), and both MACE and MACE death risk were increased along with the increasing score (Table 2). A 2.7- and 4.3-fold relative risk increase was observed for MACE and MACE death, respectively, when comparing the high- with low-risk category. When subjects were sorted according to their LDL-C concentrations and split into 4 categories in the same proportion as for the ceramide risk score, the enrichment of high-risk patients was 1.4- and 1.8-fold, respectively.

## Discussion

The ceramide analysis of the FINRISK cohort showed that distinct ceramide species associate with major cardiovascular events at the population level. In our previous work,[5,14] we have shown strong associations for ceramides and CAD outcomes in secondary prevention. These associations were further validated in this large-scale primary prevention study. Because of their association with both nonfatal incident and fatal MACE, it seems that ceramides may associate with both the development of the atherosclerotic vascular disease and its progression to vascular adverse events. The relatively strong univariate associations of ceramides with fatal events suggest that ceramides may play a role in the rupture of atherosclerotic plaques.

The clinical NRI calculations demonstrated that Cer(d18:1/18:0) holds the potential for improving the risk classification over the Framingham risk score at a population level. The majority of reclassification seemed to be mainly because of a shift from the intermediate-risk group to the low-risk group. This finding is in line with the earlier reports on risk overestimation when using the Framingham risk score.[15–20]

The combined analysis of Cer(d18:1/18:0) with CRP, HDL-C, and LDL-C was interesting and potentially applicable to improved identification of high-risk patients. In particular, the highest CRP and Cer(d18:1/18:0) quartiles identified a substantial proportion of incident MACE cases demonstrating a synergy between CRP and Cer(d18:1/18:0) (Figure 2). This marker combination could be useful for the identification of subjects in need of preventive care because of a high MACE risk. In correlation analyses, only relatively modest correlations among ceramides, CRP, and HDL-C were observed, suggesting that the observed

**Table 2. Ceramide Score and Risk for MACE and MACE Death in FINRISK 2002 Population**

| | MACE | | | | MACE Death | | | |
|---|---|---|---|---|---|---|---|---|
| | No MACE | MACE | % | Relative Risk | No Death | Death | % | Relative Risk |
| Score | | | | | | | | |
| 0–2 | 2358 | 252 | 9.7 | 1.0 | 2579 | 31 | 1.2 | 1.0 |
| 3–6 | 2854 | 448 | 13.6 | 1.4 | 3239 | 63 | 1.9 | 1.6 |
| 7–9 | 1199 | 339 | 22.0 | 2.3 | 1479 | 59 | 3.8 | 3.2 |
| 10–12 | 481 | 170 | 26.1 | 2.7 | 618 | 33 | 5.1 | 4.3 |
| LDL, mmol/L | | | | | | | | |
| ≤2.88 | 2271 | 339 | 13.0 | 1.0 | 2562 | 48 | 1.8 | 1.0 |
| 2.88–3.83 | 2807 | 495 | 15.0 | 1.2 | 3229 | 73 | 2.2 | 1.2 |
| 3.83–4.66 | 1281 | 257 | 16.7 | 1.3 | 1495 | 43 | 2.8 | 1.5 |
| ≥4.66 | 533 | 118 | 18.1 | 1.4 | 629 | 22 | 3.4 | 1.8 |

LDL indicates low-density lipoprotein; and MACE, major adverse cardiovascular event.

predictive effect of Cer(d18:1/18:0) is independent and cannot be explained by associations between these molecules. However, further studies are needed to establish the biological mechanisms for the ceramide effects and the clinical utility of the measurements.

The subanalysis of subjects who had had MACE before 2002 baseline suggested that Cer(d18:1/16:0) and Cer(d18:1/24:1) associated stronger than Cer(d18:1/18:0) with new cardiovascular events in patients with preexisting disease, which is in agreement with our earlier results on stable CAD and acute coronary syndrome patients.[4,5,14] Future mechanistic studies are needed to evaluate this difference in ceramide behavior between primary and secondary prevention. We have previously developed a ceramide score that takes into account all 3 culprit ceramides and their ratios with Cer(d18:1/24:0) to simplify the clinical use of the ceramide data. We applied the same ceramide score in the present study to the whole FINRISK 2002 cohort and observed a solid score performance at the population level.

In our previous studies in patients with established CAD, Cer(d18:1/24:0) appeared cardioprotective, whereas in the current study Cer(d18:1/24:0) was weakly associated with the increased risk of MACE and MACE death. The reason for this difference remains to be investigated in future studies. One may speculate that the different behavior of Cer(d18:1/24:0) in primary and secondary prevention studies may be related to its association with both lipoprotein particles and inflammatory processes. In fact, Cer(d18:1/24:0) behavior seems to mirror that of LDL-C. LDL-C typically associates with cardiovascular risk in primary prevention, whereas in patients with an established CAD there is an inverse relationship or no association. This has been suggested to relate to inflammatory control of hepatic LDL receptor regulation.[21] Thus, it is possible that Cer(d18:1/24:0) is biologically less active compared with other ceramide species, and in the risk prediction, it is merely a measure of lipoprotein metabolism.

By design, our study is an observational cohort study, and therefore, it is not possible to establish causality or potential treatment consequences. Thus, it is not known at the moment, whether treating cardiovascular risk on the basis of ceramide classification would produce any better clinical results than the treatments based on current risk estimation algorithms, such as the Framingham or the SCORE equation. Another limitation is the fact that our study cohort consists almost totally of whites. Thus, further studies in ethnically more diverse populations are warranted. The strengths of the study include the large FINRISK 2002 cohort, which is representative of the general Finnish population and has a long and comprehensive follow-up for cardiovascular events. Another strength is the modern and sophisticated mass spectrometry technology, which enabled the measurement of circulating ceramides in 8000 individuals with good accuracy.

In conclusion, our results show that distinct circulating ceramides are associated with the risk of incident MACE in healthy individuals. These results add insight to our understanding of the pathogenesis of MACE and suggest new options for risk estimation.





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## Disclosures

Zora Biosciences holds patents for the diagnostic use of ceramides, and R.H. and R.L. are shareholders of Zora Biosciences. R.H., R.L., M.S.-A., M.H., K.E., and D.K. are employees of Zora Biosciences. The other authors report no conflicts.

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## Highlights

- The ceramide analysis of the FINRISK cohort showed that distinct ceramide species associate with major cardiovascular events at the population level.
- Ceramides may be new biomarkers of major adverse cardiovascular event risk.
- The combination of Cer(d18:1/18:0) and CRP could be useful for the identification of subjects at high major adverse cardiovascular event risk.

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# Circulating Ceramides Predict Cardiovascular Outcomes in the Population-Based FINRISK 2002 Cohort

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# SUPPLEMENTAL MATERIAL

**Supplemental Table I:** Baseline Characteristics of the FINRISK 2002 Study Population.

| Baseline Characteristic | Men (N=3790) | Women (N=4311) |
|---|---|---|
| Total cholesterol mmol/L | 5.6 (4.9 — 6.3) | 5.4 (4.8 — 6.1) |
| HDL cholesterol mmol/L | 1.3 (1.1 — 1.5) | 1.6 (1.4 — 1.9) |
| LDL cholesterol mmol/L | 3.4 (2.9 — 4.1) | 3.2 (2.6 — 3.7) |
| Triglycerides mmol/L | 1.4 (1.0 — 2.0) | 1.1 (0.8 — 1.4) |
| Systolic blood pressure mm Hg | 135 (124 — 148) | 129 (117 — 145) |
| Age y | 50 (38 — 59) | 47 (36 — 58) |
| BMI kg/m2 | 26.8 (24.5 — 29.5) | 25.7 (22.9 — 29.3) |
| Lipid lowering medication n (%) | 321 (8.5) | 278 (6.5) |
| Antihypertensive medication n (%) | 570 (15.0) | 597 (13.9) |
| Current smoking n (%) | 1178 (31.1) | 906 (21.0) |
| Diabetes prevalence n (%) | 216 (5.7) | 232 (5.4) |
| MACE prevalence n (%) | 249 (6.6) | 147 (3.4) |

Data are median (interquartile range), or n (% from total).

**Supplemental Table II:** Cross-Validated Clinical Net Reclassification Improvement (NRI) for 13-year risk of Incident MACE.

| Baseline vs. Baseline + | Clinical NRI (%) | P-value |
|---|---|---|
| Cer(d18:1/16:0) | 5.8 (2.8 — 8.9) | $2.1\times10^{-4}$ |
| Cer(d18:1/18:0) | 7.5 (3.8 — 11.2) | $6.9\times10^{-5}$ |
| Cer(d18:1/24:0) | N/A* | N/A* |
| Cer(d18:1/24:1) | 7.2 (4.0 — 10.4) | $1.0\times10^{-5}$ |
| Cer(d18:1/16:0)/Cer(d18:1/24:0) | 5.1 (2.4 — 7.7) | $1.7\times10^{-4}$ |
| Cer(d18:1/18:0)/Cer(d18:1/24:0) | 7.1 (3.5 — 10.8) | $1.2\times10^{-4}$ |
| Cer(d18:1/24:1)/Cer(d18:1/24:0) | 5.5 (2.9 — 8.1) | $2.7\times10^{-5}$ |

**Irrelevant, as the HR of the ceramide in question is not significant.*
*MACE=major adverse cardiovascular event.*
*NRI was calculated over the 10x cross validated baseline Weibull model which used age as the time-scale, was sex stratified and included total cholesterol, HDL cholesterol, systolic blood pressure (adjusted +15mmHg for blood pressure medication), smoking, and prevalent diabetes. Clinical NRI measures reclassification among those who are in the intermediate risk group (10-year event risk 5-20%) by the baseline model.*

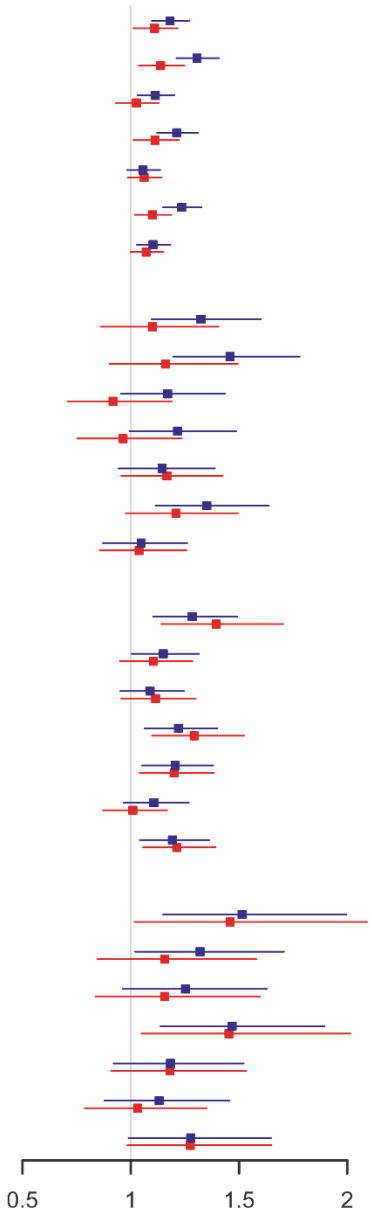
**Supplemental Table III**: Spearman rank correlation coefficients between ceramides and other lipid Markers and CRP for the whole FINRISK study population.

| | CRP | TC | TG | LDL-C | HDL-C |
|---|---|---|---|---|---|
| **Cer(d18:1/16:0)** | 0.17 | 0.62 | 0.36 | 0.57 | -0.05 |
| **Cer(d18:1/18:0)** | 0.33 | 0.51 | 0.42 | 0.50 | -0.17 |
| **Cer(d18:1/24:0)** | 0.12 | 0.62 | 0.45 | 0.59 | -0.11 |
| **Cer(d18:1/24:1)** | 0.19 | 0.56 | 0.44 | 0.53 | -0.14 |
| **Cer(d18:1/16:0)/Cer(d18:1/24:0)** | 0.03 | -0.18 | -0.23 | -0.19 | 0.10 |
| **Cer(d18:1/18:0)/Cer(d18:1/24:0)** | 0.30 | 0.03 | 0.08 | 0.05 | -0.11 |
| **Cer(d18:1/24:1)/Cer(d18:1/24:0)** | 0.11 | -0.10 | n.s. | -0.11 | -0.04 |

*Non-significant correlation (p>0.05) is marked with n.s.*

**Supplemental Figure I**: Upper panel: Unadjusted (UHR, blue) and adjusted (MHR, red) hazard ratios for incident MACE and fatal incident MACE for participants with no MACE prior to baseline (n=7,705). Adjustment for Framingham risk factors (Total-C, HDL-C, blood pressure (adjusted +15mmHg for antihypertensive medication), diabetes and smoking) and CRP. Lower panel: Unadjusted (UHR, blue) and adjusted (MHR, red) hazard ratios for recurrent MACE and fatal recurrent MACE for patients with prevalent MACE at baseline (n=396)). Adjustment for Framingham risk factors (Total-C, HDL-C, blood pressure (adjusted +15mmHg for blood pressure medication), diabetes and smoking) and CRP. Age was used as the time scale in all models and the models were stratified by sex. Therefore, the 'unadjusted' models are essentially equivalent for age- and sex-adjusted models.

| **Incident MACE (n=813)** | **UHR (95% CI)** | **MHR (95% CI)** |
|---|---|---|
| Cer(d18:1/16:0) | 1.18 (1.10, 1.27) | 1.11 (1.01, 1.22) |
| Cer(d18:1/18:0) | 1.31 (1.21, 1.41) | 1.14 (1.04, 1.25) |
| Cer(d18:1/24:0) | 1.11 (1.03, 1.20) | 1.03 (0.93, 1.13) |
| Cer(d18:1/24:1) | 1.21 (1.12, 1.31) | 1.11 (1.01, 1.22) |
| Cer(d18:1/16:0)/Cer(d18:1/24:0) | 1.06 (0.98, 1.14) | 1.06 (0.99, 1.14) |
| Cer(d18:1/18:0)/Cer(d18:1/24:0) | 1.24 (1.15, 1.33) | 1.10 (1.02, 1.19) |
| Cer(d18:1/24:1)/Cer(d18:1/24:0) | 1.10 (1.03, 1.18) | 1.07 (1.00, 1.15) |
| **Fatal incident MACE (n=116)** | | |
| Cer(d18:1/16:0) | 1.32 (1.10, 1.60) | 1.10 (0.86, 1.41) |
| Cer(d18:1/18:0) | 1.46 (1.20, 1.78) | 1.16 (0.90, 1.49) |
| Cer(d18:1/24:0) | 1.17 (0.96, 1.44) | 0.92 (0.71, 1.19) |
| Cer(d18:1/24:1) | 1.22 (0.99, 1.49) | 0.96 (0.75, 1.24) |
| Cer(d18:1/16:0)/Cer(d18:1/24:0) | 1.14 (0.94, 1.39) | 1.17 (0.96, 1.42) |
| Cer(d18:1/18:0)/Cer(d18:1/24:0) | 1.35 (1.12, 1.64) | 1.21 (0.98, 1.49) |
| Cer(d18:1/24:1)/Cer(d18:1/24:0) | 1.05 (0.87, 1.26) | 1.04 (0.86, 1.26) |
| **Recurrent MACE (n=226)** | | |
| Cer(d18:1/16:0) | 1.28 (1.10, 1.49) | 1.40 (1.14, 1.70) |
| Cer(d18:1/18:0) | 1.15 (1.01, 1.32) | 1.10 (0.95, 1.28) |
| Cer(d18:1/24:0) | 1.09 (0.95, 1.25) | 1.11 (0.96, 1.30) |
| Cer(d18:1/24:1) | 1.22 (1.06, 1.40) | 1.29 (1.10, 1.52) |
| Cer(d18:1/16:0)/Cer(d18:1/24:0) | 1.21 (1.05, 1.38) | 1.20 (1.04, 1.38) |
| Cer(d18:1/18:0)/Cer(d18:1/24:0) | 1.11 (0.97, 1.27) | 1.01 (0.87, 1.17) |
| Cer(d18:1/24:1)/Cer(d18:1/24:0) | 1.19 (1.04, 1.36) | 1.21 (1.06, 1.39) |
| **Fatal recurrent MACE (n=70)** | | |
| Cer(d18:1/16:0) | 1.51 (1.15, 1.99) | 1.46 (1.02, 2.09) |
| Cer(d18:1/18:0) | 1.32 (1.02, 1.71) | 1.16 (0.85, 1.58) |
| Cer(d18:1/24:0) | 1.25 (0.96, 1.63) | 1.16 (0.84, 1.60) |
| Cer(d18:1/24:1) | 1.47 (1.14, 1.90) | 1.45 (1.05, 2.01) |
| Cer(d18:1/16:0)/Cer(d18:1/24:0) | 1.18 (0.92, 1.52) | 1.18 (0.91, 1.53) |
| Cer(d18:1/18:0)/Cer(d18:1/24:0) | 1.13 (0.88, 1.46) | 1.03 (0.79, 1.35) |
| Cer(d18:1/24:1)/Cer(d18:1/24:0) | 1.28 (0.99, 1.65) | 1.27 (0.98, 1.65) |



# SUPPLEMENTAL MATERIAL

## Material and Methods

**Study population:** The FINRISK survey has been performed every 5 years since 1972 mainly to monitor trends in cardiovascular risk factors in Finnish population. The FINRISK 2002 study is a random sample of the population aged 25-74 years from specific geographical areas of Finland. The survey included participants from North Karelia and Northern Savo in eastern Finland, Turku and Loimaa regions in southwestern Finland, the cities of Helsinki and Vantaa in the capital region, the provinces of Northern Ostrobothnia and Kainuu in northwestern Finland and the province of Lapland in northern Finland. The sampling was stratified by sex, region and 10-year age group so that each stratum had 250 participants. In North Karelia, Lapland and the cities of Helsinki and Vantaa, the strata with 65-74 year old men and women were also sampled, each with 250 participants. The original population sample was thus 13,500 (minus 64 who had died or moved away between sampling and the survey); the overall participation rate was 65.5%, including a questionnaire and health examination where blood samples were drawn. Participants (N=8,798) were advised to fast for ≥4 hrs and avoid heavy meals earlier during the day. Serum was separated and stored in −70°C. Some analytes, e.g. conventional lipids, were measured immediately. The detailed methods are described in. [1] The study protocol was approved by the Coordinating Ethics Committee of the Helsinki University Hospital District and all participants gave written informed consent. The authors had full access to the data used in this study.

**Follow-up**: In Finland, each permanent resident is assigned a unique personal identity number at birth or after immigration, which ensures reliable linkage to the computerized health records. The Finnish national health registers ensure in practice 100% coverage of all major health events and deaths. The participants were followed through 31 Dec. 2014. Only participants who had moved permanently abroad (0.5%) prior to disease event or Dec. 31, 2014 were lost to follow-up. The MACE endpoint was defined as a composite of coronary heart disease (CHD), ischemic stroke and heart failure (HF).[2] The quality of cardiovascular endpoints in the Finnish national registers has been validated. [3–6] Prevalent events were defined as those prior to or at the baseline examination date, incident events as those thereafter. Incident events also included fatal events. For the patients who had a prevalent event, the recurrent MACE was defined as a hospitalization or a fatal recurrent MACE event, which took place after the FINRISK 2002 baseline examination and at least 28 days after the first MACE event.

**Analysis of blood samples**: The ceramides were determined by Zora Biosciences Oy from the available serum samples (N=8,101). Ceramide quantification was assessed using a validated high-throughput mass spectrometry approach [7] and performed blinded to the endpoint status or any other phenotypic data. In brief, 10 µL serum was spiked with deuterated internal standards; D7-Cer(d18:1/16:0), D7-Cer(d18:1/18:0), D7-Cer(d18:1/24:0) and D7-Cer(d18:1/24:1) and extracted in ispropanol:ethyl acetate (8:2,

v/v) using a Hamilton MICROLAB® STAR robot. The plasma levels of Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:0) and Cer(d18:1/24:1) were quantified on a QTRAP™ 6500 (SCIEX, Concord, Canada) mass spectrometer equipped with an Eksigent 100-XL UHPLC system. 5 µL sample was injected and the individual ceramides were separated on an Acquity BEH C18, 2.1×50 mm column with a particle size of 1.7 µm (Waters, Milford, MA) using a 5 min gradient comprising of 10 mM ammonium acetate in water with 0.1% formic acid (mobile phase A) and 10 mM ammonium acetate in acetonitrile:isopropanol (4:3, v/v) containing 0.1% formic acid (mobile phase B). An Acquity BEH C18, 1.7 µm VanGuard pre-column was used in front of the analytical column.

The individual ceramides were quantified in MRM mode. Quantification was assessed through calibration line samples comprising of known amounts of synthetic Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:0) and Cer(d18:1/24:1) and corresponding deuterated standards. The peak area ratios of each ceramide to its corresponding deuterated form were calculated and plotted against the added ceramide concentration and fitted by linear regression analysis. The endogenous plasma ceramide concentrations were calculated from the measured peak area ratios in the samples using the individual fitted linear regression equations. Final ceramide concentrations were presented in µM.

The precision of the assay was determined for each of the four ceramides using six quality control samples within each plate and calculating the coefficient of variation (standard deviation per mean) of these samples across all samples (<12%). The accuracy was estimated by comparing the plate-wise average concentrations of the quality control samples to the average concentration of the samples in the first two plates. For each lipid the inter-quartile range of these values was within ± 8%.

**Statistical methods:** The baseline characteristics were described using medians (inter quartile range, IQR) for continuous variables, and numbers (percentages) for categorical variables. Cox proportional hazard models with age as the time scale were used to determine associations of the ceramides with incident and fatal endpoints. Basic models were stratified for sex. Full models were additionally adjusted for the remaining Framingham risk factors [8]: total cholesterol, HDL cholesterol, systolic blood pressure (add 15mmHg for those on antihypertensive medication[9]), current smoking status and prevalent diabetes. Models with incident endpoints excluded prevalent MACE. All continuous covariates and the ceramides were log-transformed and their effects are expressed per 1SD of the transformed variable.

The improvement of the predictive ability of a model for a particular endpoint with and without the ceramides was assessed by comparing the estimates for 10-year event rates with the actual outcomes. Overfitting and dispersion of the estimates were controlled for by applying 10-fold (5-fold for fatal events) cross-validation (CV) on the Weibull proportional hazard models using age as the time scale. Model calibration was ensured with the Hosmer-Lemeshow goodness of fit test [10]. The validity of Cox proportional hazards assumptions was checked with the R-function ‘cox.zph’ (from the

'survival' package) and all models were adequate. We estimated the clinical NRI in the 5…20% risk category [11], i.e. NRI among those who were at the intermediate (5…20%) risk group when predicted by the reference model without ceramides.

The calculation of ceramide score has been established recently[12]. In brief, for each individual all three ceramide ratios and each concentration, except for Cer(d18:1/24:0), were compared to the whole study population. If the variable belonged to the 3rd quartile, the individual received +1 point, and if to the 4th quartile, +2 points. Based on the scores, the subjects were split into four risk categories (low=0-2, moderate=3-6, increased=7-9 and high=10-12). When comparing the result with LDL-C, the subjects were sorted according to their LDL-C concentrations and split into four categories in the same proportion as for the ceramide risk score.

R version 3.2.2 (R Core Team (2015), Vienna, Austria) was used for all statistical analyses. $p<0.05$ was considered as statistically significant and all tests were two-sided.

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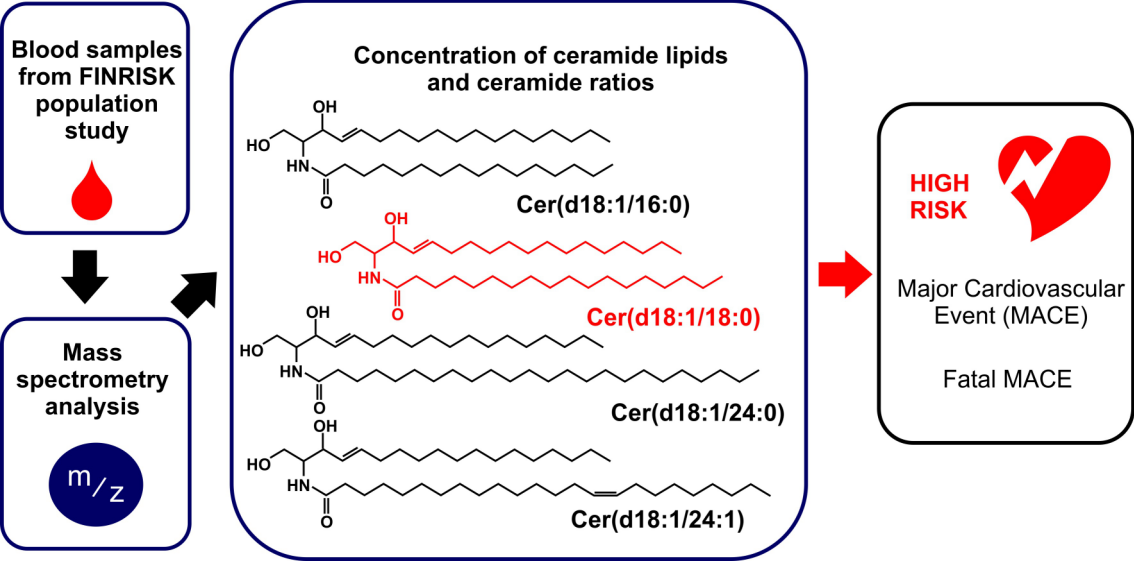
Blood samples from FINRISK population study
Mass spectrometry analysis
m/z
Concentration of ceramide lipids and ceramide ratios
Cer(d18:1/16:0)
Cer(d18:1/18:0)
Cer(d18:1/24:0)
Cer(d18:1/24:1)
HIGH RISK
Major Cardiovascular Event (MACE)
Fatal MACE

The article by Havulinna et al (*Arterioscler Thromb Vasc Biol*. 2016;36:2424-2430. DOI: 10.1161/ATVBAHA.116.307497), which published online ahead of print on October 20, 2016, was changed to be an Open Access article with a CC-BY license.

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