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 liquid chromatography–tandem mass spectrometry 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.

Key Words: cardiovascular diseases ■ ceramides ■ lipids ■ lipidomics ■ 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.
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 total cholesterol, LDL-C, HDL-C, triglycerides, ceramides, and C-reactive protein (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

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 C-reactive protein; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; MACE, major adverse cardiovascular event; TC, total cholesterol; and TG, triglycerides.

The Wilcoxon test ***P<0.001; **P<0.01 and *P<0.05.
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%; $P=6.9\times10^{-5}$), which was mainly driven by down-classification (6.9%; $P=1.2\times10^{-5}$) of risk among people 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

| Incident MACE (n=813) | UHR (95% CI) | MHR (95% CI) |
|-----------------------|--------------|--------------|
| Cer(d18:1/16:0)       | 1.16 (1.10, 1.27) | 1.14 (1.04, 1.25) |
| Cer(d18:1/18:0)       | 1.31 (1.21, 1.41) | 1.21 (1.11, 1.33) |
| 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) | 1.14 (1.04, 1.26) |
| Cer(d18:1/16:0)/Cer(d18:1/24:0) | 1.06 (0.98, 1.14) | 1.09 (1.01, 1.17) |
| Cer(d18:1/18:0)/Cer(d18:1/24:0) | 1.24 (1.15, 1.33) | 1.16 (1.08, 1.25) |
| Cer(d18:1/24:1)/Cer(d18:1/24:0) | 1.10 (1.03, 1.18) | 1.10 (1.02, 1.18) |

| Fatal incident MACE (n=116) | UHR (95% CI) | MHR (95% CI) |
|-----------------------------|--------------|--------------|
| Cer(d18:1/16:0)             | 1.32 (1.10, 1.60) | 1.13 (0.88, 1.44) |
| Cer(d18:1/18:0)             | 1.46 (1.20, 1.78) | 1.24 (0.97, 1.59) |
| Cer(d18:1/24:0)             | 1.17 (0.96, 1.44) | 0.92 (0.71, 1.18) |
| Cer(d18:1/24:1)             | 1.22 (0.99, 1.49) | 0.99 (0.78, 1.27) |
| Cer(d18:1/16:0)/Cer(d18:1/24:0) | 1.14 (0.94, 1.39) | 1.19 (0.98, 1.46) |
| Cer(d18:1/18:0)/Cer(d18:1/24:0) | 1.35 (1.12, 1.64) | 1.28 (1.04, 1.57) |
| Cer(d18:1/24:1)/Cer(d18:1/24:0) | 1.05 (0.87, 1.26) | 1.07 (0.88, 1.29) |

| Recurrent MACE (n=226) | UHR (95% CI) | MHR (95% CI) |
|------------------------|--------------|--------------|
| Cer(d18:1/16:0)        | 1.28 (1.10, 1.49) | 1.50 (1.22, 1.83) |
| Cer(d18:1/18:0)        | 1.15 (1.01, 1.32) | 1.18 (1.01, 1.37) |
| Cer(d18:1/24:0)        | 1.09 (0.95, 1.25) | 1.13 (0.97, 1.32) |
| Cer(d18:1/24:1)        | 1.22 (1.06, 1.40) | 1.34 (1.13, 1.58) |
| Cer(d18:1/16:0)/Cer(d18:1/24:0) | 1.21 (1.05, 1.38) | 1.24 (1.08, 1.43) |
| Cer(d18:1/18:0)/Cer(d18:1/24:0) | 1.11 (0.97, 1.27) | 1.08 (0.94, 1.24) |
| Cer(d18:1/24:1)/Cer(d18:1/24:0) | 1.19 (1.04, 1.36) | 1.23 (1.07, 1.41) |

| Fatal recurrent MACE (n=70) | UHR (95% CI) | MHR (95% CI) |
|-----------------------------|--------------|--------------|
| Cer(d18:1/16:0)             | 1.51 (1.15, 1.99) | 1.54 (1.07, 2.20) |
| Cer(d18:1/18:0)             | 1.32 (1.02, 1.71) | 1.23 (0.90, 1.67) |
| Cer(d18:1/24:0)             | 1.25 (0.96, 1.63) | 1.17 (0.85, 1.60) |
| Cer(d18:1/24:1)             | 1.47 (1.14, 1.90) | 1.49 (1.08, 2.05) |
| Cer(d18:1/16:0)/Cer(d18:1/24:0) | 1.18 (0.92, 1.52) | 1.23 (0.95, 1.60) |
| Cer(d18:1/18:0)/Cer(d18:1/24:0) | 1.13 (0.88, 1.46) | 1.09 (0.84, 1.42) |
| Cer(d18:1/24:1)/Cer(d18:1/24:0) | 1.28 (0.99, 1.65) | 1.30 (1.01, 1.68) |

Figure 1. 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. 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.
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).

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. 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.

Figure 2. A–C, Incident major adverse cardiovascular event (MACE) rate (%) in different concentration quartiles of Cer(d18:1/18:0), C-reactive protein (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, C-reactive protein (CRP) in Q4 vs not in Q4, and Cer(d18:1/18:0) in Q4 and CRP in Q4 vs its complement.
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, may be associated with different ceramides, we applied a previously developed ceramide risk score 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, 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.

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

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

| Score | MACE No | MACE | % | Relative Risk | MACE Death No | Death | % | Relative Risk |
|-------|---------|------|---|--------------|---------------|-------|---|--------------|
| 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 | MACE No | MACE | % | Relative Risk | MACE Death No | Death | % | Relative Risk |
|-------------|---------|------|---|--------------|---------------|-------|---|--------------|
| ≤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.
of a high MACE risk. In correlation analyses, only relatively modest correlations among ceramides, CRP, and HDL-C were observed, suggesting that the observed 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(d18:1/24:0) 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.

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 the 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 (Systematic Coronary Risk Evaluation). 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 C-reactive protein could be useful for the identification of subjects at high major adverse cardiovascular event risk.