

# Development and validation of a ceramide- and phospholipid-based cardiovascular risk estimation score for coronary artery disease patients

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## Aims

Distinct ceramide lipids have been shown to predict the risk for cardiovascular disease (CVD) events, especially cardiovascular death. As phospholipids have also been linked with CVD risk, we investigated whether the combination of ceramides with phosphatidylcholines (PCs) would be synergistic in the prediction of CVD events in patients with atherosclerotic coronary heart disease in three independent cohort studies.

## Methods and results

Ceramides and PCs were analysed using liquid chromatography–mass spectrometry (LC-MS) in three studies: WECAC (The Western Norway Coronary Angiography Cohort) ( $N=3789$ ), LIPID (Long-Term Intervention with Pravastatin in Ischaemic Disease) trial ( $N=5991$ ), and KAROLA (Langzeiterfolge der KARdiologischen Anschlussheilbehandlung) ( $N=1023$ ). A simple risk score, based on the ceramides and PCs showing the best prognostic features, was developed in the WECAC study and validated in the two other cohorts. This score was highly significant in predicting CVD mortality [multadjusted hazard ratios (HRs; 95% confidence interval) per standard deviation were 1.44 (1.28–1.63) in WECAC, 1.47 (1.34–1.61) in the LIPID trial, and 1.69 (1.31–2.17) in KAROLA]. In addition, a combination of the risk score with high-sensitivity troponin T increased the HRs to 1.63 (1.44–1.85) and 2.04 (1.57–2.64) in WECAC and KAROLA cohorts, respectively. The C-statistics in WECAC for the risk score combined with sex and age was 0.76 for CVD death. The ceramide-phospholipid risk

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score showed comparable and synergistic predictive performance with previously published CVD risk models for secondary prevention.

## Conclusion

A simple ceramide- and phospholipid-based risk score can efficiently predict residual CVD event risk in patients with coronary artery disease.

## Keywords

Ceramide • Phospholipid • Cardiovascular • Prevention • Risk • Death

## Introduction

The number of patients with atherosclerotic coronary heart disease (CHD) is increasing due to aging of the population and improved survival after CHD events. Coronary heart disease patients have been considered a rather homogenous group in terms of their risk for future cardiovascular (CV) events. However, Kaasenbrood *et al.*<sup>1</sup> have suggested that there is a substantial variation in the estimated 10-year risk of recurrent vascular events in patients with vascular disease. Hence considering all patients with vascular disease to be at similar risk may not be entirely precise, and the proposed risk stratification can be used to further optimize cardiovascular disease (CVD) prevention in high-risk patients.<sup>1</sup> This is an appealing proposition as targeted prevention of recurrent CHD events may present an opportunity for health economic gains.

In their study, Kaasenbrood *et al.*<sup>1</sup> used the previously published SMART risk score (Second Manifestations of Arterial disease) for estimation of the 10-year risk of myocardial infarction (MI), stroke, or vascular death.<sup>2</sup> The SMART risk score contains 14 clinical and laboratory variables allowing identification of vascular patients at high risk for recurrent events. This model was tested in several cohorts and, based on the findings, it was suggested that new risk estimation algorithms could allow individualized patient care also for patients with manifest CVD.<sup>1</sup> Similarly the TIMI (Thrombolysis In Myocardial Infarction) Risk Score for Secondary Prevention (TRS 2P) with 9-point risk stratification tool predicts 3-year risk for cardiovascular (CV) death, MI, and ischaemic stroke.<sup>3</sup>

We have previously demonstrated that distinct serum ceramide lipids predict CV death in patients with stable CHD and acute coronary syndrome, and are thus potential markers of residual risk.<sup>4</sup> We were keen to improve the performance of our test even further, in particular as we had previously observed in LURIC<sup>5</sup> and other unpublished studies that phosphatidylcholines (PCs) could add predictive power. The PCs were prominent candidates for test inclusion also because they could be stably incorporated into our high throughput, quality controlled, clinical ceramide assay.<sup>6</sup> Ceramides and PCs come from different biosynthetic pathways: ceramides are sphingolipids which contain a sphingosine backbone attached to a fatty acid, while PCs have a choline headgroup and two fatty acyl side chains.<sup>7</sup> These two lipid classes also differ in their distribution in lipoprotein particles.<sup>8</sup> We performed a lipidomic analysis in three prospective CHD cohorts, which allowed us to develop and validate a new ceramide- and phospholipid-based risk stratification score. Furthermore, we also investigated whether a simple combination of biomarkers would show synergy by combining the ceramide-phospholipid score with the most predictive non-lipidomic biomarker, high-sensitivity troponin-T (hsTnT).

## Methods

### Study cohorts

The data used in the current study were derived from three large clinical studies: WECAC (The Western Norway Coronary Angiography Cohort), LIPID (The Long-Term Intervention with Pravastatin in Ischaemic Disease) trial, and KAROLA (Langzeiterfolge der KARdiologischen Anschlussheilbehandlung). All studies were carried out according to the Declaration of Helsinki and approved by the local ethics committees, and are described in [Supplementary material online, Methods](#).

### Analytical methods

The sample matrix was plasma for WECAC and LIPID trial, and serum for KAROLA. For WECAC and KAROLA studies, the LC-MS/MS analyses were performed on a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500, AB Sciex, Concord, Canada) equipped with an ultra-high performance liquid chromatography (UHPLC) (Nexera-X2, Shimadzu, Kyoto, Japan). Ceramide lipids were analysed with a validated method,<sup>6</sup> and the analysis of phospholipids was performed using a global lipidomic screening platform,<sup>9</sup> in addition to a targeted method for phospholipids (PLs) referred herein as LCPL platform. Details of these methods are described in [Supplementary material online, Methods](#). The analysed phospholipids and ions used in the current study are represented in [Supplementary material online, Table S1](#). Using synthesized lipid standards it was confirmed that in the screening platform PC 36:6 corresponded to PC 14:0/22:6 lipid and in the LCPL platform PC 16:0/22:5 contained an omega3 docosapentanoic acid (DPA) side chain ([Supplementary material online, Figure S1](#)). In the LIPID trial, the ceramides and phospholipids were analysed as published recently.<sup>10</sup> In the LIPID data, the phospholipids were presented as summed composition species, and therefore, PC 38:5 was used instead of PC 16:0/22:5, PC 36:6 instead of PC 14:0/22:6, and PC 32:0 instead of PC 16:0/16:0 in the analyses.

### Statistical methods

All statistical calculations were performed using R software. The analyses were performed for two outcomes: CV death, and a composite CV event endpoint, which included CV death, MI, and stroke. For development of the new risk score, we utilized the four established ceramide molecules and ceramide ratios, which have been shown to predict CV death,<sup>4</sup> as well as those 12 phospholipids ([Supplementary material online, Table S1](#)) that significantly predicted CV events in the WECAC study. All possible lipid-to-lipid ratios for these molecules were calculated, and from these ratios and individual lipid molecules four variables were selected for the risk score in a stepwise manner ([Supplementary material online, Figure S2](#)). Based on these variables, a 0–12 point risk scoring system was developed by giving points based on the population quartiles for the variables ([Supplementary material online, Table S2](#)), and based on the scores the individuals were divided into four risk categories ([Supplementary](#)

material online, [Table S3](#)). Other statistical methods are described in detail in [Supplementary material online, Methods](#).

## Results

### Development and performance of a new prognostic lipid score

As phospholipids have shown prognostic value for CV events,<sup>10</sup> we investigated whether a ceramide test score (CERT1) could be improved by adding certain PCs. Key drivers of our selection process were analytical stability, ability to incorporate into the existing assay<sup>6</sup> and statistical robustness across several clinical cohorts. The new ceramide test score, named CERT2, was developed in the WECAC study and validated in the LIPID and KAROLA studies ([Table 1](#)), by selecting the test components in a stepwise manner ([Supplementary material online, Figure S2, Supplementary material online, Table S4](#)). The original CERT1 score consisted of three single ceramides and three ceramide/ceramide ratios, whereas the CERT2 score had one ceramide/ceramide ratio, two ceramide/PC ratios and a single PC. In general, the ceramide-PC ratio components of the CERT2 test showed higher HRs than the previously published ceramide–ceramide ratios,<sup>4</sup> across all three studies ([Supplementary material online, Table S5](#)).

The performance of the CERT2 score, in addition to other CV biomarkers, was evaluated for CV death ([Table 2](#)) and CV composite events (CV death, MI, and stroke; [Supplementary material online, Table S6](#)). For CV death, the HRs (95% confidence interval) per standard deviation (SD) for CERT2 were 1.50 (1.35–1.68) in WECAC, 1.51 (1.38–1.65) in the LIPID trial, and 1.62 (1.32–2.00) in KAROLA. For all the investigated biomarkers, the HRs for CV events were lower than for CV death [for CERT2 the HRs per SD were 1.36 (1.25–1.48) in WECAC; 1.28 (1.21–1.37) in the LIPID trial; and 1.18 (1.03–1.36) in KAROLA] ([Supplementary material online, Table S6](#)). Adjustments with traditional CV risk factors and biomarkers did not materially affect the results ([Table 2, Supplementary material online, Table S6](#)). In WECAC, for CERT2 the HR for non-CV deaths was lower than for CV deaths, and the score predicted MIs and strokes also when not combined into the CV event composite endpoint ([Supplementary material online, Table S7](#)).

### Combination of CERT2 with other biomarkers

We also wanted to test the performance of CERT2 with other biomarkers that were available for each study. We developed an additional score that combined both CERT2 and hsTnT, as in WECAC the performance of CERT2 and hsTnT appeared to be better than that of other evaluated biomarkers ([Table 2](#)), and since we had validation data available in KAROLA. This demonstrated added value over single biomarkers and CERT2-TnT score showed higher HRs than CERT2 or hsTnT alone [for CV death HRs per SD in WECAC 1.79 (1.59–2.00) and KAROLA 1.92 (1.55–2.37)] ([Table 2](#) and [Supplementary material online, Table S6](#)). In KAROLA, also another strong published secondary prevention CVD biomarker, NT-proBNP,<sup>11</sup> was measured and combination of CERT2 with this biomarker showed comparable results as combination with hsTnT ([Supplementary material online, Table S8](#)). Thus, also other

biomarkers may show synergy with CERT2, but for further analyses we focused on CERT2-TnT since we had validation data only for this combination at present.

### Performance of biomarkers in subgroups

Statin treatment might affect the performance of CVD biomarkers, but in the LIPID trial cox regression models the CERT2-treatment arm interaction did not show significant interaction ( $P=0.13$  for CV death and  $P=0.14$  for CV events). Furthermore, we investigated the performance of the markers in a statin free population, i.e. in the LIPID trial placebo arm. This analysis validated CERT2 score results obtained in the WECAC and KAROLA cohorts, and showed that conventional lipid markers show weaker prognostic value for CV death and CV events ([Supplementary material online, Table S9](#)). Furthermore, in WECAC and LIPID trial, we investigated the performance of the biomarkers separately in subjects with and without diabetes mellitus. It appeared that the cardiovascular risk prediction in subjects with diabetes mellitus is particularly challenging, as the only biomarkers showing value in this patient population were CERT2, hsTnT, or their combination ([Supplementary material online, Table S10](#)).

### Stratification of patients into risk groups

For clinical use, we divided the ceramide scores (CERT2 and CERT2-TnT) into four risk groups ranging from low- to high risk ([Supplementary material online, Table S3](#)), and in all three studies the risk for CV death and CV events systematically increased along with increasing score and risk group ([Table 3, Supplementary material online, Table S11](#)). For CV death, a 3.5- to 5.4-fold risk increase was observed in different cohorts between the lowest- and highest-risk groups for CERT2, whereas the difference for the CV events was more modest. Noteworthy, for the CERT2-TnT score a more than 10-fold increased risk was observed when comparing low- and high-risk groups. The risk increase appeared consistent along with increasing score. The largest risk increase was observed in patients who scored more than 6 points out of 12 maximum points ([Figure 1](#)). The same result was achieved in the statin free population ([Supplementary material online, Figure S3](#)) of the LIPID trial. Finally, Kaplan–Meier curves demonstrated the strong association of CERT2 and CERT2-TnT with CV death and CV events ([Figure 2](#)). For comparison, Kaplan–Meier curves for LDL-cholesterol (LDL-C) are shown in [Supplementary material online, Figure S4](#).

### CERT2 improves existing risk estimation models

Among WECAC patients, CERT2 combined with sex and age reached C-statistics of 0.76 and CERT2-TnT of 0.78 in relation to CV deaths ([Table 4](#)). For CV composite events, the C-statistics values were lower, 0.65 for CERT2 and 0.66 for CERT2-TnT, when combined with sex and age. However, these values were comparable with the SMART score C-statistics (0.64) predicting 5-year risk for CV events, and the TIMI score (0.64) predicting 3-year risk for CV events. As the CERT2 was developed based on the WECAC study, we also investigated the performance of the SMART and TIMI scores when the coefficients of the variables were based on the same cohort. In this case, the SMART variables showed higher performance

**Table 1** Clinical characteristics of the study cohorts

| Variables                           | WECAC                            |                                  |         | LIPID                               |                                     |         | KAROLA                            |                                    |         |
|-------------------------------------|----------------------------------|----------------------------------|---------|-------------------------------------|-------------------------------------|---------|-----------------------------------|------------------------------------|---------|
|                                     | No CV death                      | CV death                         | P-value | No CV death                         | CV death                            | P-value | No CV death                       | CV death                           | P-value |
| N                                   | 3449                             | 340                              | —       | 5488                                | 503                                 | —       | 918                               | 105                                | —       |
| Age (years)                         | 61 (54–68)                       | 70 (62–76)                       | 1.4E-41 | 64 (57–69)                          | 68 (63–72)                          | 3.6E-28 | 61 (54–65)                        | 64 (61–68)                         | 1.3E-07 |
| Male                                | 2455 (71)                        | 260 (76)                         | 0.045   | 4537 (83)                           | 432 (86)                            | NS      | 775 (84)                          | 88 (84)                            | NS      |
| Current smoker                      | 1095 (32)                        | 110 (32)                         | NS      | 499 (9)                             | 50 (10)                             | NS      | 48 (5)                            | 6 (6)                              | NS      |
| Statin treatment                    | 2516 (73)                        | 235 (69)                         | NS      | 2758 (50)                           | 231 (46)                            | NS      | 707 (77)                          | 66 (63)                            | 0.002   |
| Diabetes mellitus                   | 365 (11)                         | 65 (19)                          | 3.4E-06 | 438 (8)                             | 73 (15)                             | 8.0E-07 | 137 (15)                          | 40 (38)                            | 8.3E-09 |
| Hypertension                        | 1538 (45)                        | 204 (60)                         | 7.4E-08 | 2283 (42)                           | 233 (46)                            | 0.045   | 498 (54)                          | 64 (61)                            | NS      |
| Previous MI                         | 1308 (38)                        | 187 (55)                         | 1.1E-09 | 3434 (63)                           | 349 (69)                            | 0.003   | 541 (59)                          | 72 (69)                            | NS      |
| Previous stroke                     | 204 (6)                          | 47 (14)                          | 4.3E-08 | 203 (4)                             | 34 (7)                              | 0.001   | 36 (4%)                           | 8 (8)                              | NS      |
| LDL-C (mg/dL)                       | 112 (93–143)                     | 116 (96–145)                     | NS      | 150 (131–170)                       | 151 (130–172)                       | NS      | 117 (94–145)                      | 116 (94–143)                       | NS      |
| HDL-C (mg/dL)                       | 46 (39–58)                       | 46 (39–58)                       | NS      | 36 (31–41)                          | 34 (29–41)                          | 9.7E-04 | 37 (30–44)                        | 36 (30–42)                         | NS      |
| TG (mg/dL)                          | 133 (96–190)                     | 126 (94–181)                     | NS      | 141 (104–194)                       | 136 (102–185)                       | NS      | 139 (106–191)                     | 144 (113–200)                      | NS      |
| Cer(d18:1/16:0)                     | 0.254 (0.216–0.300) <sup>a</sup> | 0.273 (0.229–0.323) <sup>a</sup> | 1.2E-06 | 379 (328–441) <sup>b</sup>          | 401 (345–467) <sup>b</sup>          | 5.0E-07 | 0.277 (0.232–0.323) <sup>a</sup>  | 0.310 (0.258–0.368) <sup>a</sup>   | 6.3E-05 |
| Cer(d18:1/18:0)                     | 0.099 (0.078–0.126) <sup>a</sup> | 0.110 (0.084–0.135) <sup>a</sup> | 2.0E-04 | 143 (115–178) <sup>b</sup>          | 151 (124–195) <sup>b</sup>          | 2.1E-05 | 0.115 (0.090–0.145) <sup>a</sup>  | 0.134 (0.108–0.162) <sup>a</sup>   | 1.3E-05 |
| Cer(d18:1/24:0)                     | 2.619 (2.096–3.205) <sup>a</sup> | 2.507 (2.029–3.162) <sup>a</sup> | NS      | 3699 (3116–4381) <sup>b</sup>       | 3603 (3038–4275) <sup>b</sup>       | NS      | 2.622 (2.166–3.196) <sup>a</sup>  | 2.721 (2.222–3.277) <sup>a</sup>   | NS      |
| Cer(d18:1/24:1)                     | 1.071 (0.885–1.289) <sup>a</sup> | 1.175 (0.952–1.402) <sup>a</sup> | 3.3E-07 | 1339 (1122–1601) <sup>b</sup>       | 1414 (1184–1676) <sup>b</sup>       | 2.5E-04 | 1.241 (1.042–1.504) <sup>a</sup>  | 1.408 (1.199–1.661) <sup>a</sup>   | 1.5E-06 |
| PC 16:0/22:5                        | 15.1 (12.2–18.6) <sup>c</sup>    | 14.2 (11.5–17.8) <sup>c</sup>    | 0.003   | 14 852 (12 677–17 188) <sup>b</sup> | 13 854 (11 875–16 306) <sup>b</sup> | 1.2E-08 | 6.022 (4.812–7.528) <sup>c</sup>  | 5.865 (4.507–6.841) <sup>c</sup>   | NS      |
| PC 14:0/22:6                        | 1.181 (0.867–1.591) <sup>c</sup> | 1.112 (0.786–1.493) <sup>c</sup> | 0.003   | 343 (262–447) <sup>b</sup>          | 309 (226–406) <sup>b</sup>          | 8.6E-08 | 1.455 (0.985–2.121) <sup>c</sup>  | 1.271 (0.885–1.757) <sup>c</sup>   | 0.021   |
| PC 16:0/16:0                        | 13.7 (11.7–16.2) <sup>c</sup>    | 14.3 (11.8–17.0) <sup>c</sup>    | 0.003   | 7855 (6776–9194) <sup>b</sup>       | 7978 (6976–9462) <sup>b</sup>       | 0.022   | 9.767 (8.218–11.795) <sup>c</sup> | 11.065 (8.341–13.442) <sup>c</sup> | 0.007   |
| Cer(d18:1/16:0)/<br>Cer(d18:1/24:0) | 0.098 (0.084–0.116)              | 0.108 (0.093–0.129)              | 1.0E-12 | 0.103 (0.088–0.120)                 | 0.110 (0.094–0.130)                 | 1.8E-10 | 0.105 (0.089–0.123)               | 0.113 (0.094–0.131)                | 0.004   |
| Cer(d18:1/18:0)/<br>Cer(d18:1/24:0) | 0.039 (0.032–0.048)              | 0.042 (0.034–0.054)              | 1.6E-06 | 0.039 (0.031–0.049)                 | 0.042 (0.034–0.054)                 | 1.6E-08 | 0.043 (0.034–0.056)               | 0.047 (0.038–0.064)                | 0.002   |
| Cer(d18:1/24:1)/<br>Cer(d18:1/24:0) | 0.415 (0.338–0.509)              | 0.458 (0.386–0.565)              | 3.7E-10 | 0.364 (0.304–0.436)                 | 0.389 (0.326–0.470)                 | 5.8E-08 | 0.482 (0.391–0.572)               | 0.556 (0.444–0.627)                | 4.8E-05 |
| Cer(d18:1/16:0)/<br>PC 16:0/22:5    | 0.017 (0.013–0.021)              | 0.020 (0.015–0.024)              | 9.5E-11 | 0.026 (0.022–0.031)                 | 0.029 (0.024–0.035)                 | 3.3E-19 | 0.046 (0.035–0.060)               | 0.055 (0.045–0.066)                | 6.8E-06 |
| Cer(d18:1/18:0)/<br>PC 14:0/22:6    | 0.085 (0.058–0.125)              | 0.097 (0.068–0.148)              | 2.1E-05 | 0.414 (0.303–0.581)                 | 0.509 (0.341–0.742)                 | 4.2E-13 | 0.078 (0.049–0.126)               | 0.099 (0.074–0.162)                | 4.4E-05 |

For categorical variables absolute numbers (n) and relative proportions (%) are presented. For continuous variables, median together with the interquartile range is presented.

LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; NS, not significant; TG, triglycerides.

<sup>a</sup>μmol/L.

<sup>b</sup>Peak intensity.

<sup>c</sup>Peak area normalized with an internal standard.

**Table 2** Hazard ratios per standard deviation for the CERT scores predicting cardiovascular death, and comparison with other cardiovascular biomarkers

| Variables | WECAC                    |          |                          | LIPID   |                          |         | KAROLA                   |         |                          |         |
|-----------|--------------------------|----------|--------------------------|---------|--------------------------|---------|--------------------------|---------|--------------------------|---------|
|           | HR (95% CI) <sup>a</sup> | P-value  | HR (95% CI) <sup>b</sup> | P-value | HR (95% CI) <sup>a</sup> | P-value | HR (95% CI) <sup>b</sup> | P-value | HR (95% CI) <sup>b</sup> | P-value |
| CERT2     | 1.50 (1.35–1.68)         | 2.6E-13  | 1.44 (1.28–1.63)         | 3.1E-09 | 1.51 (1.38–1.65)         | 5.7E-19 | 1.47 (1.34–1.61)         | 2.8E-16 | 1.62 (1.32–2.00)         | 4.7E-06 |
| CERT2-TnT | 1.79 (1.59–2.00)         | <2.2E-16 | 1.63 (1.44–1.85)         | 2.2E-14 | 1.29 (1.18–1.40)         | 5.0E-09 | 1.31 (1.20–1.43)         | 3.0E-09 | 1.92 (1.55–2.37)         | 2.2E-09 |
| CERT1     | 1.27 (1.14–1.41)         | 7.9E-06  | 1.23 (1.09–1.38)         | 5.4E-04 | 1.03 (0.95–1.13)         | NS      | 1.07 (0.98–1.17)         | NS      | 1.48 (1.23–1.79)         | 4.4E-05 |
| LDL-C     | 1.05 (0.94–1.17)         | NS       | 1.15 (1.01–1.30)         | 0.032   | 0.84 (0.77–0.92)         | 3.4E-04 | 0.84 (0.76–0.94)         | 0.002   | 1.02 (0.84–1.26)         | NS      |
| HDL-C     | 0.81 (0.72–0.91)         | 3.8E-04  | 0.95 (0.84–1.07)         | NS      | 0.98 (0.89–1.08)         | NS      | 0.88 (0.79–0.98)         | 0.023   | 0.80 (0.64–1.01)         | NS      |
| TG        | 1.15 (1.04–1.27)         | 0.005    | 1.07 (0.96–1.19)         | NS      | 1.02 (0.94–1.12)         | NS      | 0.92 (0.75–1.13)         | NS      | 1.39 (1.18–1.64)         | 9.2E-05 |
| ApoB      | 1.15 (1.03–1.28)         | 0.009    | 1.33 (1.03–1.72)         | 0.031   | 0.79 (0.72–0.87)         | 1.9E-06 | 0.77 (0.64–0.93)         | 0.006   | 0.98 (0.80–1.20)         | NS      |
| ApoA1     | 0.79 (0.71–0.89)         | 8.6E-05  | 0.91 (0.74–1.12)         | NS      |                          |         |                          |         | 1.16 (1.07–1.25)         | 1.9E-04 |
| hsCRP     | 1.12 (1.05–1.20)         | 0.001    | 1.10 (1.02–1.19)         | 0.010   |                          |         |                          |         | 0.92 (0.72–1.17)         | NS      |
| hsTnT     | 1.43 (1.31–1.55)         | 2.2E-16  | 1.30 (1.19–1.43)         | 1.7E-08 |                          |         |                          |         | 1.18 (1.07–1.30)         | 0.001   |
| Lp(a)     | 1.13 (1.02–1.25)         | 0.020    | 1.13 (1.01–1.26)         | 0.029   |                          |         |                          |         |                          |         |
| TMAO      | 1.06 (0.97–1.16)         | NS       | 1.02 (0.94–1.12)         | NS      |                          |         |                          |         |                          |         |

hsCRP, high-sensitivity C-reactive protein; Lp(a), lipoprotein(a); NS, not significant; TMAO, trimethylamine N-oxide.

<sup>a</sup>Age as time scale, stratified by vitamin B intervention (WECAC), and the treatment group (LIPID).

<sup>b</sup>Additionally, adjusted for sex, statin treatment (WECAC, KAROLA), diabetes mellitus, hypertension, current smoking, previous MI, previous stroke, BMI (WECAC, LIPID TRIAL), LDL-C, HDL-C, TG, and hsCRP (WECAC, KAROLA).

(0.67) than CERT2 with sex and age. However, in all cases, of predefined and fitted scores based on WECAC, addition of CERT2 increased the C-statistics and also showed significant increase both in categorical and continuous net reclassification index (NRI). For instance, CERT2-TnT on top of the SMART score showed an overall NRI of 0.072 (95% CI 0.030–0.113) and a continuous NRI of 0.289 (0.187–0.392). Values for the addition of CERT2-TnT on top of the TIMI score were 0.123 (0.065–0.181) and 0.236 (0.119–0.354), respectively. In both cases, the categorical NRI improved more for events than for non-events, whereas the opposite was true for the continuous NRI models (Table 4). CERT2 improved C-statistics also on top of other conventional risk factors and biomarkers in all three cohorts, as shown in Supplementary material online, Table S12.

## Discussion

Our previously developed ceramide risk score (CERT) and its components have shown superior performance in comparison with traditional CV risk markers. In this study, we developed a novel and even more powerful ceramide- and phospholipid-based risk score for CHD, called CERT2, by combining three PC molecules with the previously published four ceramide lipids (Take home figure).<sup>4,12</sup> Our results showed that the molecular lipid-based risk estimation tool had improved performance metrics and can be thus used to reliably stratify CHD patients for their risk of CV events, especially CV death. The data exposed a large variability in residual risk among the study patients and the CERT2 score demonstrated an excellent and potentially clinically relevant prognostic value. We also showed that additionally improved prognostics can be achieved by combining CERT2 with other biomarkers, such as hsTnT.

The strengths of our study include the derivation/validation approach applying three independent CHD cohorts from Europe (WECAC, KAROLA) and Australia (LIPID), with a long-term follow-up. Furthermore, one of the study cohorts (LIPID) included a placebo-controlled statin trial, which allows assessment of risk associations also in a population without interfering lipid-lowering treatments. A limitation of the study was that the PCs, and partly ceramides, were analysed with a slightly different analytical methodology in all three cohorts, which did not allow us to compare the calibration of the score across the cohorts. Notably, however, within different cohorts the results replicated well despite these different methodologies. Thus, it appeared that the lipids described in this study were robust both statistically and analytically, although a fully validated mass-spectrometric laboratory method will be needed for the clinical application of CERT2.

One of the main challenges in CV risk estimation is often the modest discriminatory power of the tests and models. However, in this study a simple biomarker score (CERT2 or CERT2-TnT) together with the information on age and sex reached a C-statistics of 0.76, which can be considered fairly acceptable for multifactorial diseases like CHD. A certain level of residual risk always remains in CHD, and thus, the value of prognostic estimation is in the accurate identification of patients that exceed an acceptable risk level while on their current treatment. CERT2 improved C-statistics when added on top of the two recent risk scores, the SMART and TIMI risk scores. Also the reclassification indices improved significantly when CERT2 score

**Table 3** Risk for cardiovascular death in different CERT score groups, and with respect to LDL-C

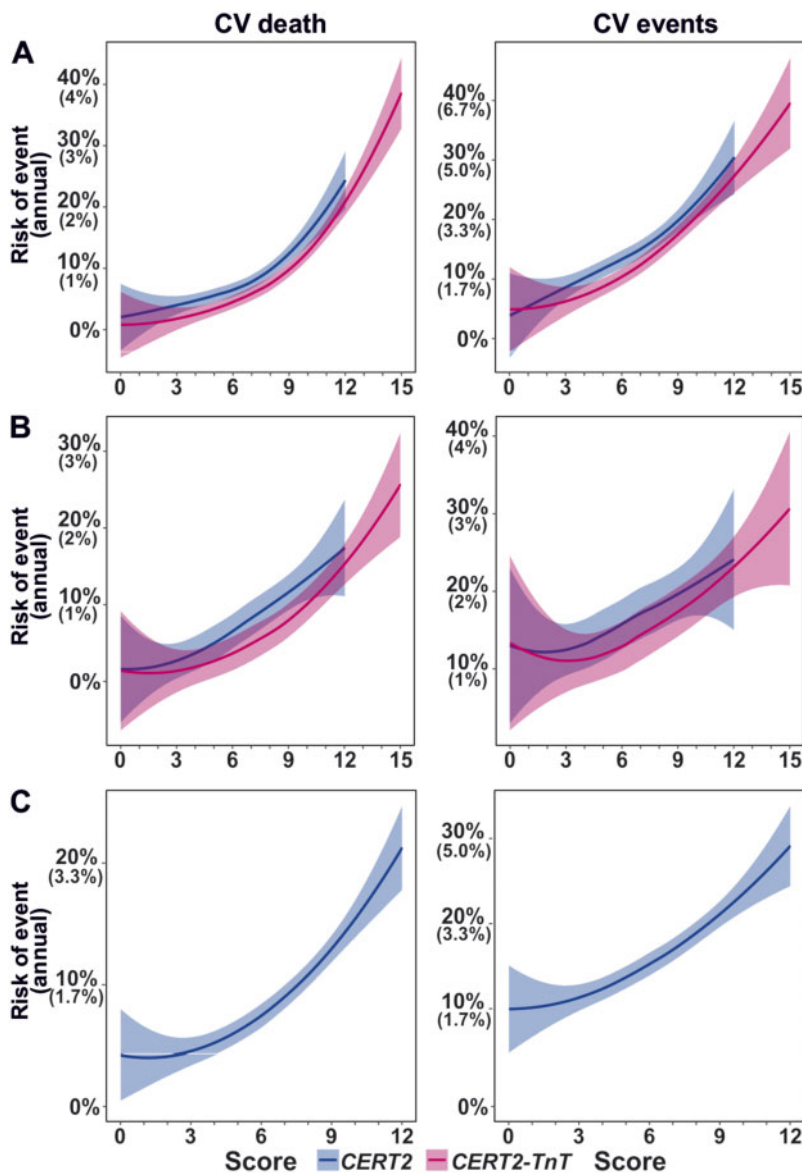
|        | CERT2 |                |          | CERT2-TnT |                |          | CERT1 |                |          | LDL-C |                |          |         |                |          |           |
|--------|-------|----------------|----------|-----------|----------------|----------|-------|----------------|----------|-------|----------------|----------|---------|----------------|----------|-----------|
|        | Group | Population (%) | Risk (%) | Group     | Population (%) | Risk (%) | Group | Population (%) | Risk (%) | Group | Population (%) | Risk (%) | mg/dL   | Population (%) | Risk (%) | Rel. risk |
| WECAC  | 0-3   | 14             | 3.5      | Ref.      | 0-4            | 17       | 2.0   | Ref.           | 0-2      | 33    | 5.1            | Ref.     | 17-81   | 14             | 7.3      | Ref.      |
|        | 4-6   | 45             | 6.4      | 1.8       | 5-7            | 39       | 5.3   | 2.7            | 3-6      | 40    | 6.3            | 1.2      | 81-124  | 45             | 7.7      | 1.1       |
|        | 7-8   | 27             | 7.8      | 2.2       | 8-10           | 29       | 9.1   | 4.6            | 7-9      | 18    | 11.1           | 2.2      | 124-162 | 27             | 8.1      | 1.1       |
|        | 9-12  | 14             | 15.1     | 4.3       | 11-15          | 11       | 20.2  | 10.2           | 10-12    | 9     | 15.5           | 3.0      | 162-402 | 14             | 6.6      | 0.9       |
| LIPID  | 0-3   | 17             | 4.3      | Ref.      | —              | —        | —     | —              | 0-2      | 34    | 5.9            | Ref.     | 46-123  | 17             | 8.8      | Ref.      |
|        | 4-6   | 41             | 6.3      | 1.5       | —              | —        | —     | —              | 3-6      | 38    | 8.7            | 1.5      | 123-156 | 41             | 7.9      | 0.9       |
|        | 7-8   | 25             | 9.9      | 2.3       | —              | —        | —     | —              | 7-9      | 18    | 8.9            | 1.5      | 156-178 | 25             | 7.9      | 0.9       |
|        | 9-12  | 17             | 15.2     | 3.5       | —              | —        | —     | —              | 10-12    | 10    | 14.8           | 2.5      | 178-273 | 17             | 9.7      | 1.1       |
| KAROLA | 0-3   | 23             | 2.6      | Ref.      | 0-4            | 22       | 1.3   | Ref.           | 0-2      | 39    | 3.5            | Ref.     | 10-92   | 23             | 7.6      | Ref.      |
|        | 4-6   | 33             | 5.4      | 2.1       | 5-7            | 29       | 3.4   | 2.5            | 3-6      | 31    | 7.0            | 2.0      | 92-121  | 32             | 6.5      | 0.9       |
|        | 7-8   | 23             | 7.8      | 3.0       | 8-10           | 27       | 8.5   | 6.4            | 7-9      | 17    | 11.8           | 3.4      | 121-148 | 23             | 7.1      | 0.9       |
|        | 9-12  | 22             | 13.8     | 5.4       | 11-15          | 21       | 16.5  | 12.4           | 10-12    | 12    | 12.7           | 3.7      | 148-286 | 22             | 5.0      | 0.7       |

For comparison, LDL-C was divided into groups (Q1-Q4) in the same proportion as CERT2. For the WECAC and KAROLA studies, the risk is for 10 years and for LIPID trial, 6 years.

was added on top of the SMART or TIMI scores, even if the coefficients of these variables were fitted for our current study. Overall, the CERT2 score appeared to provide at least as accurate information as the more comprehensive clinical scores suggesting that a simple laboratory test-based risk estimation could become a practical tool for daily clinical use. The wider adoption of various clinical scores into practice is hampered by the large number of needed variables that may not be easily available, in addition to the time consuming data entry required for these computerized algorithms. Thus, a single blood-based prognostic analysis would be expected to be more practical and also cost-efficient in daily clinical use. It is also worth noting that the most critical biomarker in the CV field, LDL-C, showed a very weak association in predicting outcomes. While LDL-C plays a central role in the aetiology of atherosclerosis, its value as a biomarker in secondary prevention appears limited across all studies. LDL-C concentration change is useful in monitoring lipid-lowering treatment response and compliance, while CERT2 may provide a more informative read-out of the patient's residual risk for secondary events. A significant finding of this study was also the lack of predictive power of traditional blood biomarkers in patients with diabetes mellitus, and particular attention should be paid to what blood chemistry is used to guide their risk assessment.

According to current guidelines, subjects with diagnosed CHD always belong to a very high-risk population.<sup>13</sup> However, in light of the present and recently published data<sup>1</sup> it appears that the risk is highly variable in this patient group. Importantly, our data show that the majority of CHD patients may have a relatively low long-term risk, <5% CV death risk in ten years. On the other hand, our data suggest that, indeed, 10-20% of all CHD patients do have an extremely high risk, i.e. 10-year risk in the range of 15-20% for CV death. CERT2 may thus add a new personalized treatment opportunity to the current practice as it appears that certain patients are well off with the current therapy, while in the others significant residual risk remains that should be addressed more aggressively. These more effective means are likely to include higher statin and other lipid-lowering medication doses, anti-thrombotic treatments, well maintained blood pressure targets, and intense lifestyle coaching to improve adherence to given therapies. As an example, in the PREDIMED trial, participants with high-ceramide concentrations were shown to benefit from Mediterranean diet,<sup>14</sup> and in another study, PCSK9 inhibition was shown to benefit especially those subjects at high CV risk.<sup>15</sup> In the future, biomarker guided risk stratification may be part of a more cost-efficient personalized treatment of CHD.

Ceramides are known to affect several CHD-related processes, including LDL aggregation and uptake, endothelial dysfunction and multiple inflammatory processes.<sup>4</sup> Interestingly, two out of three phospholipids selected in CERT2 contained polyunsaturated fatty acids (PUFAs). Both DPA (22:5) and docosahexaenoic acid (22:6) belong to the omega3 (*n* - 3) fatty acid series, which may have several positive CHD-related effects, such as anti-thrombotic and anti-inflammatory effects, in addition to influencing overall lipid metabolism, heart rate, blood pressure, and endothelial function.<sup>16</sup> However, dietary and *n* - 3 fatty acid supplementation trials have shown discordant results in the prevention of CV events,<sup>17</sup> which is exemplified also by two recent large trials: while *n* - 3 fatty acids were not shown to affect statistically significantly major cardiovascular events in the VITAL trial,<sup>18</sup> high-dose eicosapentaenoic acid ethyl ester



**Figure 1** Risk curves for CERT2 and CERT2-TnT scores in (A) WECAC, (B) KAROLA, and (C) LIPID studies. The risks are calculated for 10 years in KAROLA and WECAC cardiovascular death, and 6 years in LIPID trial and WECAC cardiovascular events. In Y-axes, the annual risk rates are shown in parentheses.

investigated in the REDUCE-IT showed a 25% relative reduction of cardiovascular events.<sup>19</sup> An interesting area for further studies is to test whether CERT2 could identify those subjects that will benefit from *n*-3 fatty acid supplementation.

In conclusion, CERT2 score offers an easy to use tool for residual risk estimation in CHD. This validated risk stratification tool may be used to further improve development of personalized management of CHD, which should be tested in future prospective studies.

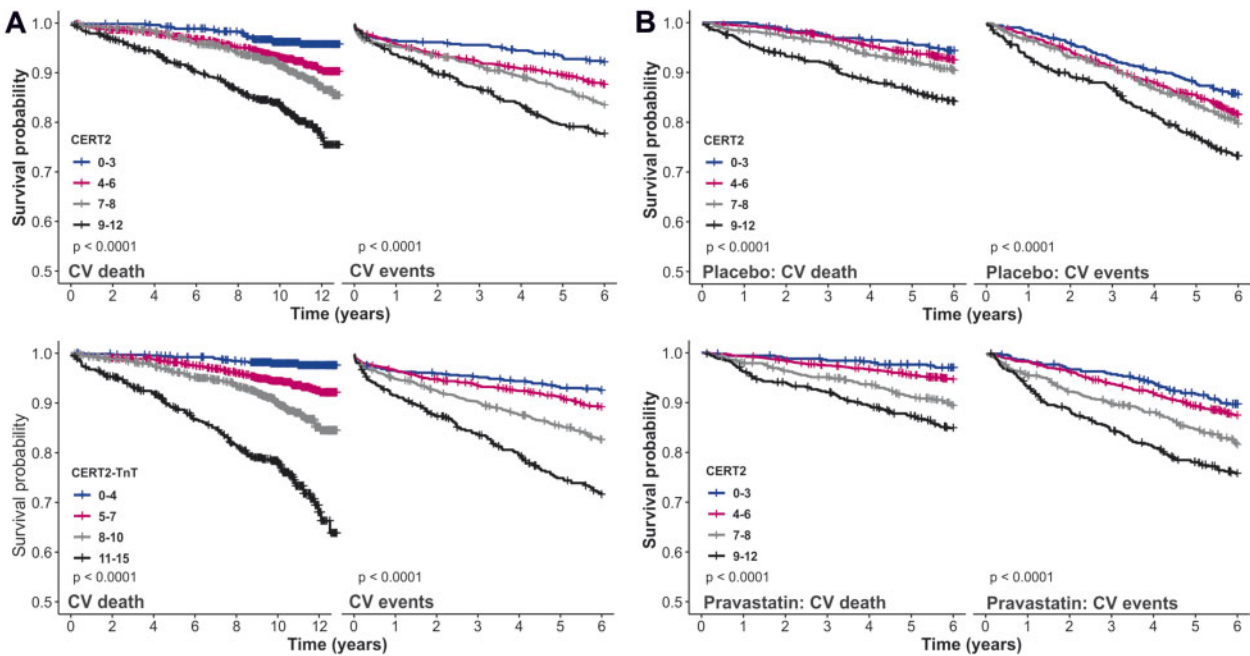
## Supplementary material

Supplementary material is available at *European Heart Journal* online.

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**Conflict of interest:** Zora Biosciences Oy holds patent disclosures related for the diagnostic and prognostic use of ceramides and phospholipids in CVD. M.H., M.L., D.K., A.J., and R.L. are employees and R.L. a shareholder of Zora Biosciences Oy. P.J.M. is an inventor of a patent (WO 2011/063470) that has been licensed to Zora Biosciences Oy. Outside the submitted work, A.M.T. reports personal fees from Amgen,



**Figure 2** A Kaplan–Meier curves for (A) CERT2 and CERT2-TnT scores in WECAC and (B) CERT2 in the placebo and pravastatin treatment arms in the LIPID trial.

**Table 4** C-statistics together with categorical and continuous NRI values for selected variables in the WECAC study

| Variables                    | Endpoint <sup>c</sup> | C-stat | Categorical NRI <sup>d</sup> |            |                | Continuous NRI      |            |                |
|------------------------------|-----------------------|--------|------------------------------|------------|----------------|---------------------|------------|----------------|
|                              |                       |        | Overall (95% CI)             | Events (%) | Non-events (%) | Overall (95% CI)    | Events (%) | Non-events (%) |
| Sex + age                    | CV death              | 0.719  |                              |            |                |                     |            |                |
| Sex + age + CERT2            | CV death              | 0.755  | 0.158 (0.040–0.276)          | 12.3       | 3.5            | 0.316 (0.137–0.495) | 11.5       | 20.1           |
| Sex + age + CERT2-TnT        | CV death              | 0.776  | 0.196 (0.069–0.322)          | 14.2       | 5.4            | 0.581 (0.408–0.754) | 31.7       | 26.4           |
| Sex + age                    | CV event              | 0.620  |                              |            |                |                     |            |                |
| Sex + age + CERT2            | CV event              | 0.645  | 0.049 (0.005–0.094)          | 2.2        | 2.7            | 0.276 (0.178–0.374) | 12.5       | 15.1           |
| Sex + age + CERT2-TnT        | CV event              | 0.660  | 0.092 (0.041–0.142)          | 4.1        | 5.1            | 0.348 (0.250–0.447) | 16.4       | 18.4           |
| SMART score                  | CV event              | 0.644  |                              |            |                |                     |            |                |
| SMART score + CERT2          | CV event              | 0.652  | 0.047 (0.011–0.083)          | 7.3        | –2.7           | 0.146 (0.044–0.248) | 4.5        | 10.1           |
| SMART score + CERT2-TnT      | CV event              | 0.661  | 0.072 (0.030–0.113)          | 11.4       | –4.2           | 0.289 (0.187–0.392) | 13.0       | 15.9           |
| SMART variables <sup>a</sup> | CV event              | 0.666  |                              |            |                |                     |            |                |
| SMART variables + CERT2      | CV event              | 0.675  | 0.053 (0.015–0.090)          | 2.5        | 2.8            | 0.172 (0.074–0.271) | 7.7        | 9.6            |
| SMART variables + CERT2-TnT  | CV event              | 0.685  | 0.090 (0.047–0.132)          | 5.1        | 3.9            | 0.269 (0.170–0.368) | 13.4       | 13.5           |
| TIMI score                   | CV event              | 0.644  |                              |            |                |                     |            |                |
| TIMI score + CERT2           | CV event              | 0.658  | 0.069 (0.023–0.115)          | 10.1       | –3.1           | 0.140 (0.023–0.256) | 0.6        | 13.3           |
| TIMI score + CERT2-TnT       | CV event              | 0.672  | 0.123 (0.065–0.181)          | 11.3       | 1.0            | 0.236 (0.119–0.354) | 6.3        | 17.3           |
| TIMI variables <sup>b</sup>  | CV event              | 0.659  |                              |            |                |                     |            |                |
| TIMI variables + CERT2       | CV event              | 0.669  | 0.061 (0.021–0.102)          | 2.3        | 3.9            | 0.152 (0.036–0.268) | 9.7        | 5.5            |
| TIMI variables + CERT2-TnT   | CV event              | 0.679  | 0.081 (0.026–0.136)          | 1.7        | 6.4            | 0.238 (0.121–0.355) | 10.3       | 13.5           |

<sup>a</sup>SMART score variables: age, age<sup>2</sup>, sex, current smoking, systolic blood pressure, diabetes mellitus, prior MI, prior stroke, PAD, years since first diagnosis of vascular disease, HDL-C, TC, GFR, GFR<sup>2</sup>, and hsCRP.

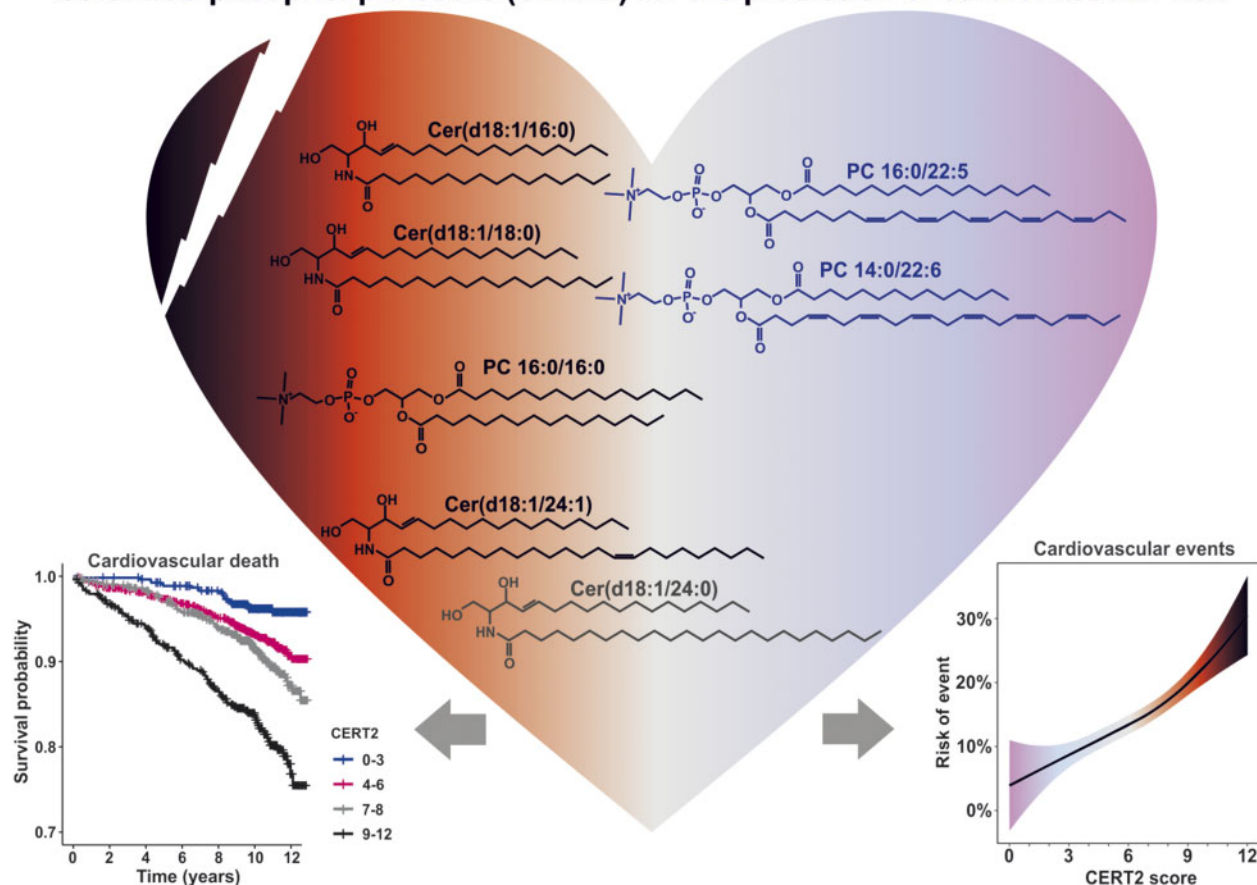
<sup>b</sup>TIMI score variables: age, current smoking, hypertension, diabetes mellitus, prior CABG, prior stroke, PAD, heart failure, and GFR.

<sup>c</sup>For TIMI score and variables, the calculations were performed for 3 years. All other results are shown for 5-year risk.

<sup>d</sup>The categorical NRI cut-offs were 2.5% and 7.5% for 5-year risk of CV death, 5% and 15% for 5-year risk of CV events, and 5% and 10% for 3-year risk of CV events.



## Ceramide-phospholipid score (CERT2) for the prediction of cardiovascular risk



**Take home figure** Distinct ceramide and phosphatidylcholine lipids were used in the derivation of a novel risk score (CERT2) that determines the risk for cardiovascular events and death, as illustrated in the figure for the WECAC study cohort.

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