

SUPPLEMENTARY MATERIAL

Hilvo et al., “Development and validation of a ceramide- and phospholipid-based cardiovascular risk estimation score for coronary artery disease patients”

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Supplementary Methods

Description of study cohorts

WECAC. The Western Norway Coronary Angiography Cohort (WECAC) consists of 5,209 adult patients who underwent coronary angiography at Haukeland University Hospital, Bergen, or Stavanger University Hospital, Stavanger, in Norway between January 2000 and April 2004.¹ The primary aim of WECAC was to investigate prognostic cardiovascular biomarkers and it included 3,090 patients who were randomised to participate in the Western Norway B vitamin Intervention Trial (WENBIT; ClinicalTrials.gov number NCT00354081), to investigate the effect of B vitamin supplementation on mortality and cardiovascular events.² The trial did not find an effect of treatment with folic acid/vitamin B12 or B16 on cardiovascular events or total mortality.² Only patients examined due to suspected stable angina pectoris were included in the current investigation, and subjects with hsTnT >30 were excluded, leaving a total of 3882 participants for the final analyses. Endpoint data was obtained from discharge or death certificate diagnoses (according to the ICD-10 system) by linkage to the CVD-NOR project (www.cvdnor.no).³ Median follow-up time for CV death (N=340) was 10.3 years, and due to shorter follow-up data on non-fatal events, the analyses of CV events (N=526) was restricted to 6 years.

LIPID trial. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) trial was initiated in 1989 to investigate the effects of pravastatin on death from CHD in patients with a history of myocardial infarction or unstable angina and a broad range of initial cholesterol levels.⁴ In brief, the patients (aged 31-75 years) were randomised 3-36 months after MI or hospitalization for unstable angina, with total cholesterol 4.0-7.0 mmol/L (155-271 mg/dL). 5991 out of total 9014 patients had lipidomic measurements available: of these 3002 received placebo and 2989 pravastatin treatment. Patients were recruited during years 1992-1997. The follow-up time was censored at 6.0 years, to restrict the analyses to the randomized controlled phase of the study.

KAROLA. KAROLA (Langzeiterfolge der KARdiOLogischen Anschlussheilbehandlung) is a prospective cohort study in which 1,206 patients with CHD aged 30-70 years participated in an in-hospital rehabilitation program between January 1999 and May 2000 in two co-operating clinics (Schwabenland-Klinik, Isny and Klinik am Südpark, Bad Nauheim, Germany). Beside quality of life and other patient reported information, the occurrence of secondary cardiovascular events was evaluated by contacting the primary care physicians, or in case of death, the respective Health Department. The composite outcome (N=224) was defined as either CVD as the main cause of death (as stated in the death certificate, N=105), non-fatal myocardial infarction (MI), or ischemic cerebrovascular event (stroke). The follow-up time of the study was 13 years. Details of the study design have been published elsewhere.⁵

Analytical methods of phospholipids in WECAC and KAROLA

Phospholipids in KAROLA were extracted using a modified Folch extraction⁶, while in WECAC the extraction was based on a previously described method⁷. Briefly, 10 µl of 10 mM 2,6-di-tert-butyl-4-methylphenol (BHT) in methanol was added to 10 µL of samples, followed by 20 µl of internal standards (Avanti Polar Lipids Inc., Alabaster, AL) and 300 µl of chloroform:methanol (2:1, v:v) (Sigma-Aldrich GmbH, Steinheim, Germany). Samples were mixed and sonicated in a water bath for 10 min, followed by a 40 minute incubation and centrifugation (15 min at 5700xg). The upper phase was transferred and evaporated under nitrogen. Extracted lipids were resuspended in 100 µl of water saturated butanol and sonicated in a water bath for 5 minutes. 100 µl of methanol was added to the samples before the extracts were centrifuged for 5 min at 3500xg, and finally the supernatants were transferred to the mass spectrometry analysis plates for analysis.

Chromatographic separation and mass spectrometric analyses for the screening platform have been described previously⁸. The chromatography for the targeted LCPL platform was performed on Acquity BEH

C18, 2.1 × 50 mm id. 1.7 µm column (Waters, Massachusetts, USA). Mobile phases consisted of (A) 10 mM ammonium acetate in LC-MS grade water with 0.1% formic acid, and (B) 10 mM ammonium acetate in acetonitrile:2-propanol (3:4, V/V) with 0.1% formic acid (FA). The following LC gradient was used: 0.5 min at 75% B, linear increase of B from 75% to 80% in 10 min, 80% to 100% B in 0.1 min, 2.4 min at 100% B, 100% to 65% B in 0.1 min and 1 min equilibration at 65% prior to the next injection. Flow rate was 500 µl/min and column temperature 60°C. Injection volume of all samples was 2 µl. Negative ionization was used in the MS analysis, and the data were collected using multiple reaction monitoring (MRM). MS settings were the same for all phospholipids in the analysis, and the conditions were as following: curtain gas (nitrogen) 25, collision activated dissociation (nitrogen) 6, temperature 300 C, gas 1: 50, gas2: 50, IHE:ON, spray voltage -4500 V, declustering potential -100 V, entry potential -10 V, collision exit potential -20 V. 10ms dwell time and collision energy of -50 was applied to all analytes. Results were processed using Analyst 1.6 and MultiQuant 3.0 software (AB Sciex, Concord, Canada).

Statistical methods

Baseline characteristics of the cohorts are described using medians (interquartile range, IQR) for continuous variables, and numbers (percentages) for categorical variables. The statistical significance between subjects experiencing CV death versus not was evaluated by Wilcoxon rank-sum test or chi-squared test, as appropriate. In all analyses, $p > 0.05$ was considered as statistically non-significant (n.s.). Correction for multiple hypothesis testing was not performed in the discovery cohort (WECAC), because the results were validated in two additional cohorts, and the focus of the study was on model performance. Cox proportional hazard regression analyses were used to determine associations with CV death and all CV events. WECAC models were stratified by the vitamin intervention group, and LIPID trial by treatment arm, and the adjusting variables for each cohort are listed as footnotes of the result tables. For adjusting variables, we selected traditional CV risk factors. In the models baseline age was used as time scale, and the effects are expressed per standard deviation (SD). Risk and calibration curves were constructed with *ggplot2* package using loess method. When constructing risk tables and curves, the observations were right-censored at 10 years for WECAC and KAROLA and at 6 years for the LIPID trial. Kaplan-Meier curves were constructed using the *survminer* package.

C-statistics calculations for Cox regression models together with the net reclassification index (NRI) calculations were performed using the *Hmisc* package. For TIMI score the results were calculated for 3-year risk following a previous publication⁹ and for SMART score for 5 years by dividing the 10-year risk by a factor of 2¹⁰. The TIMI score variables included congestive heart failure, hypertension, age 75 years or older, diabetes mellitus, prior stroke and coronary artery bypass grafting, peripheral artery disease (PAD), low eGFR and current smoking. For SMART score, the only variable missing was the information on abdominal aortic aneurysm, and thus the variables included age, sex, diabetes mellitus, current smoking, systolic blood pressure, TC, HDL-C, hsCRP, GFR, years since first vascular events, and history of cerebrovascular, coronary and PAD. In addition to predefined scores, we also calculated the results for the risk score variables fitted in the WECAC study, in order not to favour our risk score, which was developed based on WECAC. For NRI, both continuous and categorical versions were calculated. For the 5-year risk of CV events (sex combined with age and SMART score models), we chose 5% and 15% categorical NRI cut-offs, by taking half of the published lowest and highest risk cut-offs¹¹. For 5-year risk of CV death, we used half of these values, i.e. 2.5% and 7.5%. For 3-year risk of CV events the cut-offs were 5% and 10%, which were estimated based on the WECAC 3-year mean event risk for the three TIMI score risk categories⁹.

References for Supplementary Methods

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Supplementary Tables

Supplementary Table S1. List of phospholipids that were used for the CERT2 score development. The left panel shows all the phospholipids that were used as potential candidates. From these, only those showing significant association with CV events in WECAC were selected for final score development. These lipids together with analytical details are shown in the right panel.

ALL LIPIDS USED FOR SELECTION			SIGNIFICANT LIPIDS USED FOR SCORE DEVELOPMENT				
LCPL	Screening		Method	Phospholipid	Analyte	Q1 [M+HCOO] ⁻ (m/z)	Q3 [M-H] ⁻ (m/z)
PC 14:0/18:1	PC 28:0	PC 36:5	LCPL	PC 16:0/16:0	PC 32:0-16:0	778.6	255.2
PC 14:0/18:2	PC 28:1	PC 36:6	LCPL	PC 16:0/18:3	PC 34:3-16:0	800.6	255.2
PC 16:0/16:0	PC 30:0	PC 37:1	LCPL		PC 34:3-18:3	800.6	277.2
PC 16:0/16:1	PC 30:1	PC 37:2	LCPL	PC 16:0/20:4	PC 36:4-16:0	826.6	255.2
PC 16:0/18:1	PC 30:2	PC 37:3	LCPL		PC 36:4-20:4	826.6	303.2
PC 16:0/18:2	PC 31:0	PC 37:4	LCPL	PC 16:0/22:5	PC 38:5-16:0	852.6	255.2
PC 16:0/18:3	PC 31:1	PC 38:0	LCPL		PC 38:5-22:5	852.6	329.2
PC 16:0/20:3	PC 32:1	PC 38:1	LCPL	PC 16:0/22:6	PC 38:6-16:0	850.6	255.2
PC 16:0/20:4	PC 32:2	PC 38:2	LCPL		PC 38:6-22:6	850.6	327.2
PC 16:0/20:5	PC 32:3	PC 38:3	LCPL	PC 16:1/18:2	PC 34:3-16:1	800.6	253.2
PC 16:0/22:5	PC 33:1	PC 38:4	LCPL		PC 34:3-18:2	800.6	279.2
PC 16:0/22:6	PC 33:2	PC 38:5	LCPL	PC 17:0/20:4	PC 37:4-17:0	840.6	269.2
PC 16:1/18:0	PC 33:3	PC 38:6	LCPL		PC 37:4-20:4	840.6	303.2
PC 16:1/18:2	PC 34:0	PC 39:0	LCPL	PC 18:0/20:3	PC 38:3-18:0	856.6	283.3
PC 17:0/18:1	PC 34:1	PC 39:2	LCPL		PC 38:3-20:3	856.6	305.2
PC 17:0/18:2	PC 34:2	PC 39:4	LCPL	PC 18:0/20:4	PC 38:4-18:0	854.6	283.3
PC 17:0/20:4	PC 34:3	PC 39:6	LCPL		PC 38:4-20:4	854.6	303.2
PC 18:0/18:1	PC 35:0	PC 40:1	LCPL	PC 18:1/18:1	PC 36:2-18:1	830.6	281.2
PC 18:0/18:2	PC 35:1	PC 40:2					
PC 18:0/20:3	PC 35:2	PC 40:3					
PC 18:0/20:4	PC 35:3	PC 40:4					
PC 18:0/20:5	PC 36:0	PC 40:6					
PC 18:1/18:1	PC 36:1	PC 40:8					
PC 18:1/18:2	PC 36:2						
PC 18:2/18:2	PC 36:3						
			Method	Phospholipid	Analyte	Q1 [M+H] ⁺ (m/z)	Q3 [M+H] ⁺ (m/z)
			Screening	PC 36:6	PC 36:6	778.5	184.1
			Screening	PC 40:8	PC 40:8	830.6	184.1

Supplementary Table S2. Calculation of the CERT1, CERT2 and CERT2-TnT / CERT2-BNP scores. For each individual, the score variables were compared with the whole study population and the risk points were given based on which quartile (Q1) the individual belonged to. For instance for CERT2, a value in the lowest quartile gave 0 points, whereas the highest quartile resulted in 3 points. The points were summed up, to have a scoring system of 0-12 points for CERT1 and CERT2, and 0-15 points for CERT2-TnT / CERT2-BNP.

CERT1	Q1	Q2	Q3	Q4	Score
Cer(d18:1/16:0)	0	0	1	2	0-12
Cer(d18:1/18:0)	0	0	1	2	
Cer(d18:1/24:1)	0	0	1	2	
Cer(d18:1/16:0) / Cer(d18:1/24:0)	0	0	1	2	
Cer(d18:1/18:0) / Cer(d18:1/24:0)	0	0	1	2	
Cer(d18:1/24:1) / Cer(d18:1/24:0)	0	0	1	2	
CERT2	Q1	Q2	Q3	Q4	Score
Cer(d18:1/24:1) / Cer(d18:1/24:0)	0	1	2	3	0-12
Cer(d18:1/16:0) / PC 16:0/22:5	0	1	2	3	
Cer(d18:1/16:0) / PC 14:0/22:6	0	1	2	3	
PC 16:0/16:0	0	1	2	3	
CERT2-TnT / CERT2-BNP	Q1	Q2	Q3	Q4	Score
Cer(d18:1/24:1) / Cer(d18:1/24:0)	0	1	2	3	0-15
Cer(d18:1/16:0) / PC 16:0/22:5	0	1	2	3	
Cer(d18:1/16:0) / PC 14:0/22:6	0	1	2	3	
PC 16:0/16:0	0	1	2	3	
hsTnT / NT-proBNP	0	1	2	3	

Supplementary Table S3. Risk groups for the risk scores.

Risk group	CERT1	CERT2	CERT2-TnT
Low risk	0-2	0-3	0-4
Moderate risk	3-6	4-6	5-7
Increased risk	7-9	7-8	8-10
High risk	10-12	9-12	11-15

Supplementary Table S4. Hazard ratios (HRs) per standard deviation of logarithmised components of the ceramide and phospholipid based risk score when all the components were used simultaneously to predict CV death or composite CV event endpoint in WECAC and LIPID studies. The models were stratified by vitamin B intervention (WECAC) or statin treatment (LIPID).

Variable	WECAC				LIPID			
	CV death		CV events		CV death		CV events	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Cer(d18:1/24:1) / Cer(d18:1/24:0)	1.42 (1.27, 1.58)	1.7E-10	1.23 (1.13, 1.34)	1.8E-06	1.25 (1.14, 1.36)	2.1E-06	1.10 (1.04, 1.16)	7.9E-04
Cer(d18:1/16:0) / PC 16:0/22:5	1.50 (1.32, 1.71)	8.0E-10	1.23 (1.11, 1.36)	1.1E-04	1.41 (1.27, 1.57)	3.3E-10	1.19 (1.11, 1.27)	2.4E-07
Cer(d18:1/18:0) / PC 14:0/22:6	1.11 (0.98, 1.26)	0.096	1.14 (1.03, 1.27)	0.009	1.15 (1.03, 1.28)	0.011	1.12 (1.05, 1.20)	5.4E-04
PC 16:0/16:0	1.25 (1.12, 1.39)	5.4E-05	1.12 (1.03, 1.23)	0.010	1.11 (1.02, 1.21)	0.019	1.09 (1.03, 1.15)	0.002

Supplementary Table S5. Hazard ratios (HR) per standard deviation for the CERT1 and CERT2 score components, with CV death and all CV events as endpoints. n.s., not significant.

Variable	CERT		WECAC				LIPID				KAROLA			
	1	2	CV death		CV events		CV death		CV events		CV death		CV events	
			HR (95% CI) ^a	p-value	HR (95% CI) ^a	p-value	HR (95% CI) ^a	p-value	HR (95% CI) ^a	p-value	HR (95% CI) ^a	p-value	HR (95% CI) ^a	p-value
Cer(d18:1/16:0)	X		1.35 (1.20, 1.51)	3.1E-07	1.21 (1.11, 1.33)	3.8E-05	1.24 (1.13, 1.35)	2.2E-06	1.17 (1.10, 1.24)	5.1E-07	1.34 (1.08, 1.66)	0.007	1.14 (0.99, 1.32)	n.s.
Cer(d18:1/18:0)	X		1.23 (1.10, 1.38)	2.1E-04	1.16 (1.06, 1.27)	0.001	1.25 (1.14, 1.37)	1.1E-06	1.20 (1.12, 1.28)	2.1E-08	1.29 (1.05, 1.58)	0.015	1.09 (0.94, 1.25)	n.s.
Cer(d18:1/24:1)	X		1.20 (1.08, 1.35)	0.001	1.13 (1.03, 1.24)	0.007	1.14 (1.04, 1.25)	0.004	1.12 (1.05, 1.19)	3.5E-04	1.47 (1.18, 1.83)	7.1E-04	1.13 (0.98, 1.31)	n.s.
PC 16:0/22:5			0.90 (0.80, 1.00)	0.058	0.95 (0.87, 1.04)	0.291	0.80 (0.73, 0.88)	1.1E-06	0.91 (0.85, 0.97)	0.003	0.96 (0.79, 1.18)	n.s.	0.95 (0.83, 1.09)	n.s.
PC 14:0/22:6			0.79 (0.71, 0.89)	4.5E-05	0.85 (0.78, 0.93)	5.1E-04	0.80 (0.73, 0.87)	8.6E-07	0.90 (0.85, 0.96)	0.001	0.90 (0.73, 1.10)	n.s.	0.89 (0.78, 1.02)	n.s.
PC 16:0/16:0		X	1.16 (1.04, 1.31)	0.010	1.12 (1.03, 1.23)	0.013	1.11 (1.02, 1.21)	0.019	1.10 (1.03, 1.16)	0.003	1.24 (1.01, 1.52)	0.043	1.03 (0.89, 1.18)	n.s.
Cer(d18:1/16:0) / Cer(d18:1/24:0)	X		1.24 (1.11, 1.39)	1.2E-04	1.15 (1.05, 1.26)	0.002	1.31 (1.20, 1.43)	1.2E-09	1.15 (1.08, 1.22)	1.0E-05	1.20 (0.97, 1.49)	n.s.	1.07 (0.92, 1.24)	n.s.
Cer(d18:1/18:0) / Cer(d18:1/24:0)	X		1.21 (1.08, 1.36)	0.001	1.14 (1.04, 1.25)	0.004	1.32 (1.20, 1.44)	5.1E-09	1.18 (1.11, 1.26)	2.3E-07	1.18 (0.96, 1.46)	n.s.	1.03 (0.89, 1.19)	n.s.
Cer(d18:1/24:1) / Cer(d18:1/24:0)	X	X	1.12 (1.00, 1.26)	0.047	1.08 (0.99, 1.18)	0.088	1.21 (1.11, 1.33)	3.1E-05	1.11 (1.04, 1.18)	0.001	1.32 (1.05, 1.66)	0.016	1.06 (0.91, 1.22)	n.s.
Cer(d18:1/16:0) / PC 16:0/22:5		X	1.38 (1.23, 1.55)	4.3E-08	1.20 (1.10, 1.31)	8.1E-05	1.43 (1.31, 1.56)	2.1E-15	1.23 (1.16, 1.31)	5.0E-11	1.25 (1.02, 1.53)	0.033	1.15 (1.00, 1.32)	n.s.
Cer(d18:1/18:0) / PC 14:0/22:6		X	1.37 (1.23, 1.53)	2.3E-08	1.24 (1.14, 1.36)	1.3E-06	1.38 (1.26, 1.50)	5.0E-13	1.22 (1.14, 1.29)	3.3E-10	1.23 (1.01, 1.51)	0.040	1.14 (1.00, 1.32)	n.s.

^aAdjustments: Age as time scale, sex, statin treatment (WECAC, KAROLA), diabetes mellitus, hypertension, current smoking, previous MI, previous stroke, stratified by vitamin B intervention (WECAC) and treatment group (LIPID). The data were log transformed.

Supplementary Table S6. Hazard ratios (HR) per standard deviation for the CERT scores predicting all CV events (CV death, MI, stroke), and comparison with other CV biomarkers. n.s., not significant.

Variable	WECAC				LIPID				KAROLA			
	HR (95% CI) ^a	p-value	HR (95% CI) ^b	p-value	HR (95% CI) ^a	p-value	HR (95% CI) ^b	p-value	HR (95% CI) ^a	p-value	HR (95% CI) ^b	p-value
CERT2	1.36 (1.25, 1.48)	5.0E-12	1.29 (1.17, 1.42)	1.7E-07	1.28 (1.21, 1.37)	3.2E-15	1.25 (1.17, 1.33)	1.2E-11	1.18 (1.03, 1.36)	0.018	1.20 (1.01, 1.41)	0.034
CERT2-TnT	1.53 (1.40, 1.68)	<2.2E-16	1.39 (1.26, 1.54)	7.9E-11					1.31 (1.14, 1.50)	1.7E-04	1.34 (1.13, 1.58)	7.5E-04
CERT1	1.24 (1.14, 1.35)	8.5E-07	1.18 (1.08, 1.30)	4.1E-04	1.18 (1.11, 1.25)	5.5E-08	1.16 (1.09, 1.24)	3.6E-06	1.14 (1.00, 1.30)	0.046	1.13 (0.97, 1.32)	n.s.
LDL-C	1.03 (0.94, 1.12)	n.s.	1.13 (1.02, 1.24)	0.015	1.05 (0.99, 1.12)	n.s.	1.09 (1.02, 1.16)	0.007	1.09 (0.95, 1.24)	n.s.	1.13 (0.98, 1.30)	n.s.
HDL-C	0.83 (0.75, 0.91)	8.8E-05	0.94 (0.85, 1.04)	n.s.	0.88 (0.82, 0.94)	9.9E-05	0.91 (0.84, 0.98)	0.011	0.81 (0.69, 0.94)	0.006	0.85 (0.72, 1.00)	n.s.
TG	1.15 (1.07, 1.24)	2.8E-04	1.08 (1.00, 1.18)	n.s.	1.07 (1.01, 1.13)	0.031	1.00 (0.93, 1.07)	n.s.	1.21 (1.07, 1.37)	0.003	1.04 (0.86, 1.25)	n.s.
ApoB	1.09 (1.00, 1.18)	n.s.	0.99 (0.80, 1.23)	n.s.	1.08 (1.02, 1.15)	0.009	0.95 (0.82, 1.09)	n.s.				
ApoA1	0.84 (0.76, 0.92)	1.6E-04	0.94 (0.80, 1.10)	n.s.	0.88 (0.83, 0.94)	1.5E-04	0.92 (0.81, 1.05)	n.s.				
hsCRP	1.08 (1.02, 1.15)	0.013	1.06 (0.99, 1.14)	n.s.					0.93 (0.79, 1.08)	n.s.	0.90 (0.75, 1.07)	n.s.
hsTnT	1.35 (1.26, 1.45)	4.1E-16	1.23 (1.14, 1.34)	1.8E-07					1.12 (1.05, 1.20)	6.7E-04	1.12 (1.04, 1.21)	0.003
Lp(a)	1.12 (1.03, 1.21)	0.010	1.10 (1.01, 1.19)	0.034								
TMAO	1.04 (0.96, 1.12)	n.s.	1.01 (0.93, 1.10)	n.s.								

^aAge as time scale, stratified by vitamin B intervention (WECAC), treatment group (LIPID); ^badditionally 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, hsCRP (WECAC, KAROLA)

Supplementary Table S7. Cause-specific hazard ratios (HR) per standard deviation for the biomarkers predicting CV death (N=340), non-CV death (N=430), CV events (N=526), all MIs (N=406) and strokes (N=100). Age was used as timescale and the models were stratified by vitamin B intervention. n.s., not significant.

Variable	CV death		Non-CV death		CV events		Total MI		Stroke	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
CERT2	1.50 (1.35, 1.68)	2.6E-13	1.18 (1.07, 1.30)	0.001	1.36 (1.25, 1.48)	5.0E-12	1.29 (1.17, 1.43)	4.7E-07	1.43 (1.17, 1.74)	4.1E-04
CERT2-TnT	1.79 (1.59, 2.00)	<2.2E-16	1.27 (1.14, 1.40)	6.1E-06	1.53 (1.40, 1.68)	<2.2E-16	1.47 (1.32, 1.63)	4.4E-13	1.62 (1.32, 2.00)	5.0E-06
CERT1	1.27 (1.14, 1.41)	7.9E-06	1.10 (1.00, 1.20)	n.s.	1.24 (1.14, 1.35)	8.5E-07	1.23 (1.12, 1.36)	2.9E-05	1.20 (0.99, 1.46)	n.s.
LDL-C	1.05 (0.94, 1.17)	n.s.	0.97 (0.88, 1.08)	n.s.	1.03 (0.94, 1.12)	n.s.	0.95 (0.86, 1.06)	n.s.	1.27 (1.06, 1.52)	0.010
HDL-C	0.81 (0.72, 0.91)	3.8E-04	0.73 (0.66, 0.82)	2.3E-08	0.83 (0.75, 0.91)	8.8E-05	0.81 (0.72, 0.90)	1.7E-04	0.95 (0.77, 1.16)	n.s.
TG	1.15 (1.04, 1.27)	0.005	1.14 (1.04, 1.25)	0.004	1.15 (1.07, 1.24)	2.8E-04	1.15 (1.05, 1.25)	0.002	1.17 (0.98, 1.38)	n.s.
ApoB	1.15 (1.03, 1.28)	0.009	1.06 (0.97, 1.17)	n.s.	1.09 (1.00, 1.18)	n.s.	1.02 (0.92, 1.13)	n.s.	1.27 (1.06, 1.52)	0.010
ApoA1	0.79 (0.71, 0.89)	8.6E-05	0.75 (0.67, 0.83)	2.0E-08	0.84 (0.76, 0.92)	1.6E-04	0.82 (0.73, 0.91)	2.0E-04	1.03 (0.84, 1.25)	n.s.
Lpa	1.13 (1.02, 1.25)	0.020	0.97 (0.88, 1.08)	n.s.	1.12 (1.03, 1.21)	0.010	1.14 (1.04, 1.25)	0.007	0.90 (0.72, 1.12)	n.s.
hsCRP	1.12 (1.05, 1.20)	0.001	1.15 (1.10, 1.21)	2.0E-08	1.08 (1.02, 1.15)	0.013	1.05 (0.97, 1.14)	n.s.	1.12 (0.99, 1.28)	n.s.
TMAO	1.06 (0.97, 1.16)	n.s.	1.02 (0.94, 1.12)	n.s.	1.04 (0.96, 1.12)	n.s.	1.01 (0.92, 1.11)	n.s.	0.97 (0.80, 1.18)	n.s.
hsTnT	1.43 (1.31, 1.55)	2.2E-16	1.19 (1.09, 1.30)	4.9E-05	1.35 (1.26, 1.45)	4.1E-16	1.37 (1.26, 1.49)	5.7E-14	1.30 (1.11, 1.53)	0.001

Supplementary Table S8. Hazard ratios (HR) per standard deviation for the CERT2 score combined with NT-proBNP (BNP) predicting CV death and CV events in KAROLA. The results for hsTnT are shown for comparison.

Variable	CV death				CV events			
	HR (95% CI) ^a	p-value	HR (95% CI) ^b	p-value	HR (95% CI) ^a	p-value	HR (95% CI) ^b	p-value
CERT2	1.62 (1.32, 2.00)	4.7E-06	1.69 (1.31, 2.17)	4.2E-05	1.18 (1.03, 1.36)	0.018	1.20 (1.01, 1.41)	0.034
hsTnT	1.16 (1.07, 1.25)	1.9E-04	1.18 (1.07, 1.30)	0.001	1.12 (1.05, 1.20)	6.7E-04	1.12 (1.04, 1.21)	0.003
BNP	1.16 (1.08, 1.24)	2.8E-05	1.18 (1.08, 1.28)	2.0E-04	1.13 (1.07, 1.20)	1.6E-05	1.14 (1.07, 1.21)	9.8E-05
CERT2-TnT	1.92 (1.55, 2.37)	2.2E-09	2.04 (1.57, 2.64)	8.6E-08	1.31 (1.14, 1.50)	1.7E-04	1.34 (1.13, 1.58)	7.5E-04
CERT2-BNP	1.97 (1.59, 2.45)	9.5E-10	2.05 (1.58, 2.67)	8.4E-08	1.34 (1.16, 1.54)	5.2E-05	1.39 (1.17, 1.64)	1.5E-04

^aAge as time scale, ^badditionally adjusted for sex, statin treatment, diabetes mellitus, hypertension, current smoking, previous MI, previous stroke, LDL-C, HDL-C, TG, and hsCRP.

Supplementary Table S9. Hazard ratios (HR) per standard deviation for the CERT scores and other cardiovascular biomarkers in the LIPID trial placebo and pravastatin treatment arms. n.s., not significant.

	CV death					CV events			
	Variable	HR (95% CI) ^a	p-value	HR (95% CI) ^b	p-value	HR (95% CI) ^a	p-value	HR (95% CI) ^b	p-value
Placebo	CERT2	1.46 (1.29, 1.66)	1.6E-09	1.44 (1.27, 1.64)	1.1E-08	1.27 (1.17, 1.38)	2.4E-08	1.24 (1.14, 1.35)	9.1E-07
	CERT1	1.28 (1.14, 1.43)	3.8E-05	1.32 (1.16, 1.49)	1.1E-05	1.17 (1.08, 1.27)	1.4E-04	1.16 (1.06, 1.26)	0.001
	LDL-C	1.02 (0.90, 1.15)	n.s.	1.04 (0.92, 1.18)	n.s.	1.06 (0.98, 1.15)	n.s.	1.10 (1.01, 1.20)	0.027
	HDL-C	0.88 (0.78, 1.00)	n.s.	0.86 (0.74, 1.00)	n.s.	0.89 (0.82, 0.97)	0.008	0.90 (0.82, 1.00)	0.041
	TG	0.94 (0.82, 1.07)	n.s.	0.86 (0.74, 1.00)	0.046	1.05 (0.97, 1.13)	n.s.	0.99 (0.90, 1.08)	n.s.
	ApoB	0.99 (0.88, 1.12)	n.s.	0.97 (0.74, 1.27)	n.s.	1.08 (0.99, 1.17)	n.s.	0.94 (0.78, 1.12)	n.s.
	ApoA1	0.82 (0.72, 0.94)	0.004	0.80 (0.62, 1.03)	n.s.	0.89 (0.82, 0.97)	0.010	0.95 (0.80, 1.13)	n.s.
Pravastatin	CERT2	1.66 (1.46, 1.90)	2.7E-14	1.60 (1.40, 1.83)	4.6E-12	1.38 (1.26, 1.51)	7.7E-12	1.33 (1.21, 1.46)	2.7E-09
	CERT1	1.36 (1.20, 1.54)	1.4E-06	1.37 (1.20, 1.57)	2.6E-06	1.24 (1.13, 1.35)	3.7E-06	1.22 (1.11, 1.34)	3.7E-05
	LDL-C	1.03 (0.91, 1.18)	n.s.	1.10 (0.96, 1.25)	n.s.	1.01 (0.92, 1.11)	n.s.	1.06 (0.96, 1.17)	n.s.
	HDL-C	0.80 (0.69, 0.92)	0.002	0.79 (0.67, 0.94)	0.006	0.87 (0.79, 0.96)	0.004	0.90 (0.80, 1.00)	n.s.
	TG	1.00 (0.88, 1.15)	n.s.	0.87 (0.74, 1.02)	n.s.	1.07 (0.98, 1.17)	n.s.	0.99 (0.89, 1.10)	n.s.
	ApoB	1.04 (0.91, 1.18)	n.s.	0.89 (0.64, 1.23)	n.s.	1.07 (0.98, 1.17)	n.s.	0.99 (0.79, 1.23)	n.s.
	ApoA1	0.77 (0.67, 0.88)	1.6E-04	0.78 (0.59, 1.03)	n.s.	0.88 (0.80, 0.97)	0.008	0.94 (0.78, 1.14)	n.s.

^aAdjustments: Age as time scale; ^badditionally adjusted for sex, diabetes mellitus, hypertension, current smoking, previous MI, previous stroke

Supplementary Table S10. Hazard ratios (HR) per standard deviation for the CERT scores and other biomarkers predicting CV death and all CV events (CV death, MI, stroke) in subjects with and without diabetes mellitus. n.s., not significant.

		Diabetes mellitus				No diabetes mellitus				
	Variable	HR (95% CI) ^a	p-value	HR (95% CI) ^b	p-value	HR (95% CI) ^a	p-value	HR (95% CI) ^b	p-value	
WECAC	CV death	CERT2	1.52 (1.17, 1.98)	0.002	1.45 (1.06, 1.99)	0.019	1.46 (1.29, 1.65)	7.5E-10	1.43 (1.25, 1.63)	1.6E-07
		CERT2-TnT	1.92 (1.42, 2.58)	1.8E-05	1.75 (1.24, 2.46)	0.001	1.71 (1.51, 1.94)	2.1E-17	1.59 (1.39, 1.82)	2.2E-11
		CERT1	1.39 (1.07, 1.81)	0.015	1.39 (1.04, 1.87)	0.027	1.22 (1.08, 1.37)	0.001	1.20 (1.06, 1.37)	0.005
		LDL-C	1.17 (0.91, 1.50)	n.s.	1.30 (0.98, 1.71)	n.s.	1.06 (0.94, 1.19)	n.s.	1.11 (0.97, 1.28)	n.s.
		HDL-C	0.81 (0.62, 1.05)	n.s.	0.95 (0.73, 1.23)	n.s.	0.83 (0.73, 0.94)	0.004	0.93 (0.80, 1.07)	n.s.
		TG	1.06 (0.81, 1.38)	n.s.	0.94 (0.69, 1.29)	n.s.	1.11 (0.97, 1.26)	n.s.	1.05 (0.91, 1.21)	n.s.
		ApoB	1.22 (0.95, 1.55)	n.s.	1.28 (0.68, 2.40)	n.s.	1.13 (1.01, 1.27)	0.036	1.31 (0.99, 1.75)	n.s.
		ApoA1	0.78 (0.60, 1.02)	n.s.	1.03 (0.61, 1.74)	n.s.	0.81 (0.71, 0.92)	0.001	0.94 (0.75, 1.19)	n.s.
		Lpa	1.04 (0.80, 1.37)	n.s.	1.02 (0.77, 1.35)	n.s.	1.16 (1.04, 1.29)	0.008	1.18 (1.05, 1.32)	0.006
		hsCRP	1.11 (0.93, 1.33)	n.s.	1.13 (0.92, 1.39)	n.s.	1.11 (1.02, 1.20)	0.010	1.09 (1.00, 1.18)	0.047
		TMAO	0.76 (0.52, 1.12)	n.s.	0.67 (0.45, 1.00)	n.s.	1.10 (1.01, 1.21)	0.025	1.09 (1.00, 1.19)	n.s.
	hsTnT	1.68 (1.35, 2.08)	2.5E-06	1.58 (1.22, 2.04)	4.7E-04	1.36 (1.24, 1.49)	2.0E-10	1.25 (1.13, 1.38)	2.1E-05	
	CV events	CERT2	1.19 (0.95, 1.49)	n.s.	1.02 (0.78, 1.33)	n.s.	1.36 (1.24, 1.50)	1.8E-10	1.32 (1.18, 1.46)	3.6E-07
		CERT2-TnT	1.46 (1.14, 1.85)	0.002	1.21 (0.91, 1.59)	n.s.	1.52 (1.37, 1.67)	2.2E-16	1.41 (1.26, 1.57)	8.4E-10
		CERT1	1.11 (0.89, 1.37)	n.s.	0.97 (0.76, 1.24)	n.s.	1.24 (1.13, 1.37)	4.5E-06	1.22 (1.10, 1.35)	1.4E-04
		LDL-C	1.18 (0.96, 1.45)	n.s.	1.20 (0.94, 1.54)	n.s.	1.03 (0.93, 1.13)	n.s.	1.13 (1.02, 1.26)	0.020
		HDL-C	0.83 (0.65, 1.05)	n.s.	0.98 (0.77, 1.24)	n.s.	0.84 (0.75, 0.93)	7.9E-04	0.89 (0.80, 1.00)	n.s.
		TG	1.22 (1.01, 1.47)	0.039	1.18 (0.96, 1.45)	n.s.	1.09 (0.99, 1.20)	n.s.	1.04 (0.93, 1.15)	n.s.
		ApoB	1.24 (1.01, 1.54)	0.044	1.00 (0.61, 1.66)	n.s.	1.06 (0.96, 1.17)	n.s.	0.98 (0.77, 1.25)	n.s.
		ApoA1	0.81 (0.64, 1.01)	n.s.	0.88 (0.59, 1.29)	n.s.	0.85 (0.77, 0.94)	0.002	0.99 (0.83, 1.18)	n.s.
		Lpa	1.01 (0.81, 1.26)	n.s.	0.94 (0.75, 1.19)	n.s.	1.13 (1.03, 1.23)	0.008	1.11 (1.01, 1.22)	0.023
		hsCRP	1.08 (0.87, 1.35)	n.s.	1.05 (0.82, 1.36)	n.s.	1.08 (1.01, 1.15)	0.024	1.06 (0.99, 1.14)	n.s.
TMAO		0.84 (0.61, 1.15)	n.s.	0.73 (0.51, 1.03)	n.s.	1.06 (0.98, 1.15)	n.s.	1.05 (0.97, 1.14)	n.s.	
hsTnT	1.60 (1.32, 1.95)	2.4E-06	1.47 (1.16, 1.85)	0.001	1.30 (1.20, 1.41)	1.9E-10	1.20 (1.10, 1.31)	6.2E-05		
LIPID	CV death	CERT2	1.50 (1.17, 1.92)	0.001	1.59 (1.23, 2.04)	3.3E-04	1.49 (1.36, 1.65)	6.0E-16	1.46 (1.32, 1.61)	1.3E-13
		CERT1	1.17 (0.94, 1.47)	n.s.	1.32 (1.04, 1.68)	0.023	1.30 (1.18, 1.42)	2.3E-08	1.32 (1.20, 1.45)	1.8E-08
		LDL-C	1.12 (0.89, 1.42)	n.s.	1.17 (0.91, 1.51)	n.s.	1.04 (0.95, 1.15)	n.s.	1.06 (0.97, 1.17)	n.s.
		HDL-C	0.86 (0.67, 1.10)	n.s.	0.76 (0.55, 1.03)	n.s.	0.85 (0.77, 0.94)	0.002	0.85 (0.75, 0.95)	0.005
		TG	0.86 (0.66, 1.12)	n.s.	0.76 (0.54, 1.05)	n.s.	0.98 (0.88, 1.08)	n.s.	0.90 (0.80, 1.01)	n.s.
		ApoB	1.01 (0.79, 1.28)	n.s.	0.93 (0.49, 1.78)	n.s.	1.02 (0.93, 1.13)	n.s.	0.91 (0.73, 1.13)	n.s.
		ApoA1	0.88 (0.69, 1.12)	n.s.	1.23 (0.75, 2.03)	n.s.	0.78 (0.71, 0.87)	3.6E-06	0.71 (0.58, 0.87)	0.001
	CV events	CERT2	1.32 (1.11, 1.56)	0.002	1.38 (1.16, 1.64)	3.7E-04	1.27 (1.19, 1.35)	3.5E-12	1.23 (1.15, 1.31)	4.3E-09
		CERT1	1.17 (1.00, 1.38)	n.s.	1.23 (1.03, 1.46)	0.021	1.17 (1.10, 1.25)	1.3E-06	1.15 (1.08, 1.23)	4.9E-05
		LDL-C	1.14 (0.97, 1.35)	n.s.	1.18 (0.99, 1.41)	n.s.	1.06 (0.99, 1.13)	n.s.	1.08 (1.01, 1.16)	0.024
		HDL-C	0.99 (0.84, 1.17)	n.s.	0.98 (0.80, 1.20)	n.s.	0.87 (0.81, 0.94)	1.4E-04	0.89 (0.82, 0.97)	0.005
		TG	0.99 (0.83, 1.18)	n.s.	1.04 (0.84, 1.29)	n.s.	1.06 (0.99, 1.13)	n.s.	1.00 (0.92, 1.07)	n.s.
		ApoB	1.12 (0.94, 1.32)	n.s.	1.09 (0.70, 1.68)	n.s.	1.08 (1.01, 1.15)	0.026	0.92 (0.79, 1.07)	n.s.
		ApoA1	1.08 (0.92, 1.26)	n.s.	1.40 (1.01, 1.95)	0.041	0.86 (0.80, 0.92)	1.8E-05	0.85 (0.74, 0.97)	0.020

^aAge as time scale, stratified by vitamin B intervention (WECAC), treatment group (LIPID); ^badditionally adjusted for sex, statin treatment (WECAC), diabetes mellitus, hypertension, current smoking, previous MI, previous stroke, BMI, LDL-C, HDL-C, TG and hsCRP (WECAC)

Supplementary Table S11. Risk for CV events (CV death, MI and stroke) in different CERT score groups, and with respect to LDL-C. For comparison, LDL-C was divided into groups in the same proportion as CERT2. The risk is 10 years for KAROLA, and 6 years for WEACAC and LIPID trial.

	CERT2				CERT2-TnT				CERT1				LDL-C			
	Group	Population	Risk	Rel.risk	Group	Population	Risk	Rel.risk	Group	Population	Risk	Rel.risk	mg/dL	Population	Risk	Rel.risk
WEACAC	0-3	14 %	7.6 %	Ref.	0-4	17 %	7.3 %	Ref.	0-2	33 %	9.8 %	Ref.	17-81	14 %	15.9 %	Ref.
	4-6	45 %	12.1 %	1.6	5-7	39 %	10.6 %	1.4	3-6	40 %	13.6 %	1.4	81-124	45 %	12.7 %	0.8
	7-8	27 %	16.2 %	2.1	8-10	29 %	17.1 %	2.3	7-9	18 %	17.4 %	1.8	124-162	27 %	15.7 %	1.0
	9-12	14 %	21.7 %	2.8	11-15	11 %	27.4 %	3.7	10-12	9 %	23.3 %	2.4	162-402	14 %	12.6 %	0.8
LIPID	0-3	17 %	12.3 %	Ref.	-	-	-	-	0-2	34 %	14.2 %	Ref.	46-123	17 %	17.9 %	Ref.
	4-6	41 %	15.3 %	1.2	-	-	-	-	3-6	38 %	17.7 %	1.2	123-156	41 %	16.1 %	0.9
	7-8	25 %	19.0 %	1.5	-	-	-	-	7-9	18 %	18.6 %	1.3	156-178	25 %	17.6 %	1.0
	9-12	17 %	25.0 %	2.0	-	-	-	-	10-12	10 %	24.7 %	1.7	178-273	17 %	19.4 %	1.1
KAROLA	0-3	23 %	12.4 %	Ref.	0-4	22 %	10.6 %	Ref.	0-2	39 %	12.9 %	Ref.	10-92	23 %	15.6 %	Ref.
	4-6	33 %	14.4 %	1.2	5-7	29 %	11.8 %	1.1	3-6	31 %	16.8 %	1.3	92-121	32 %	14.0 %	0.9
	7-8	23 %	18.2 %	1.5	8-10	27 %	19.9 %	1.9	7-9	17 %	19.1 %	1.5	121-148	23 %	17.4 %	1.1
	9-12	22 %	20.0 %	1.6	11-15	21 %	22.5 %	2.1	10-12	12 %	19.8 %	1.5	148-286	22 %	15.0 %	1.0

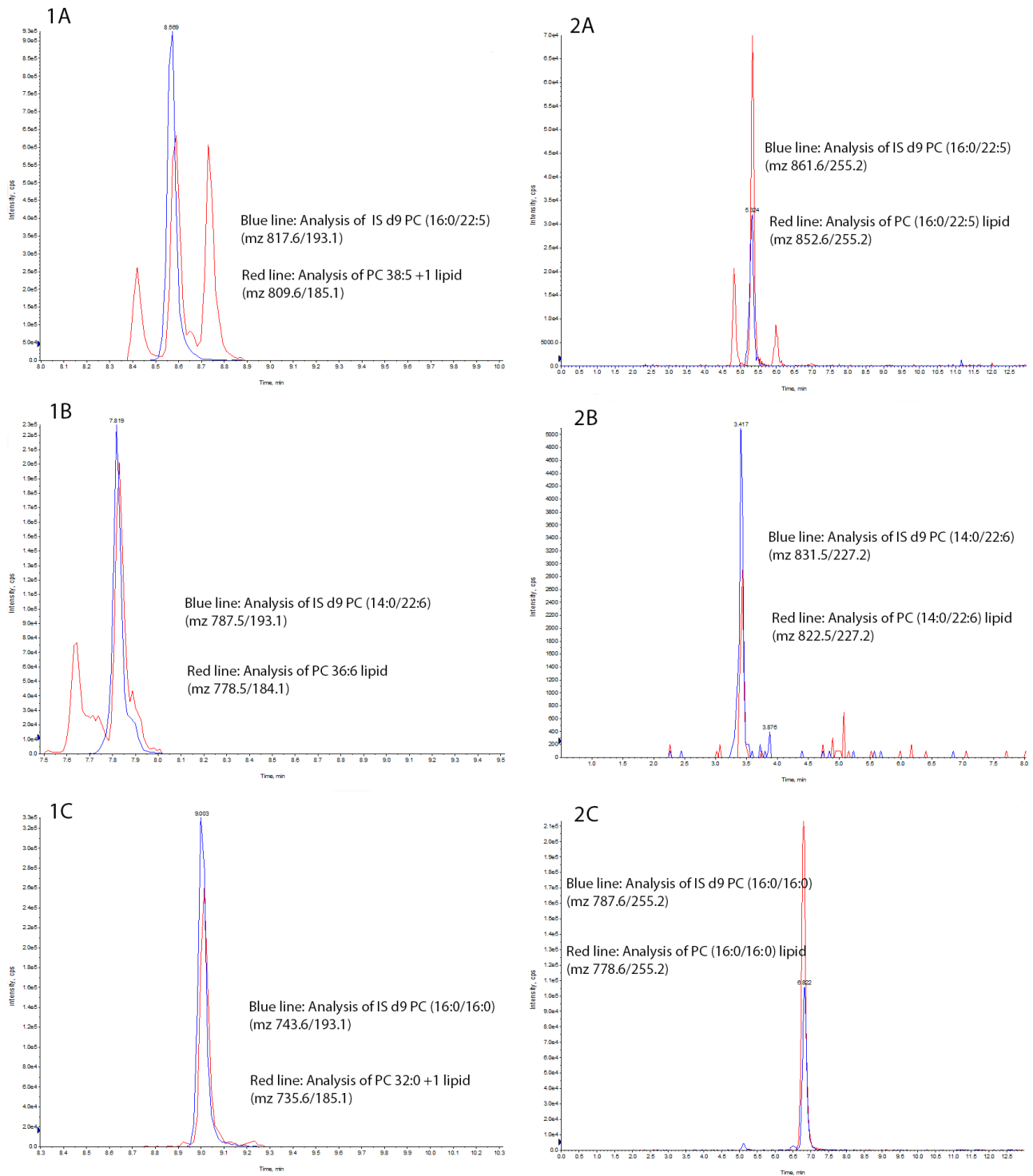
Ref. = reference category.

Supplementary Table S12. Comparison of CERT2 C-statistics with conventional CV risk factors and biomarkers. IN WECAC, the models were stratified for vitamin B intervention and in LIPID for statin treatment arm.

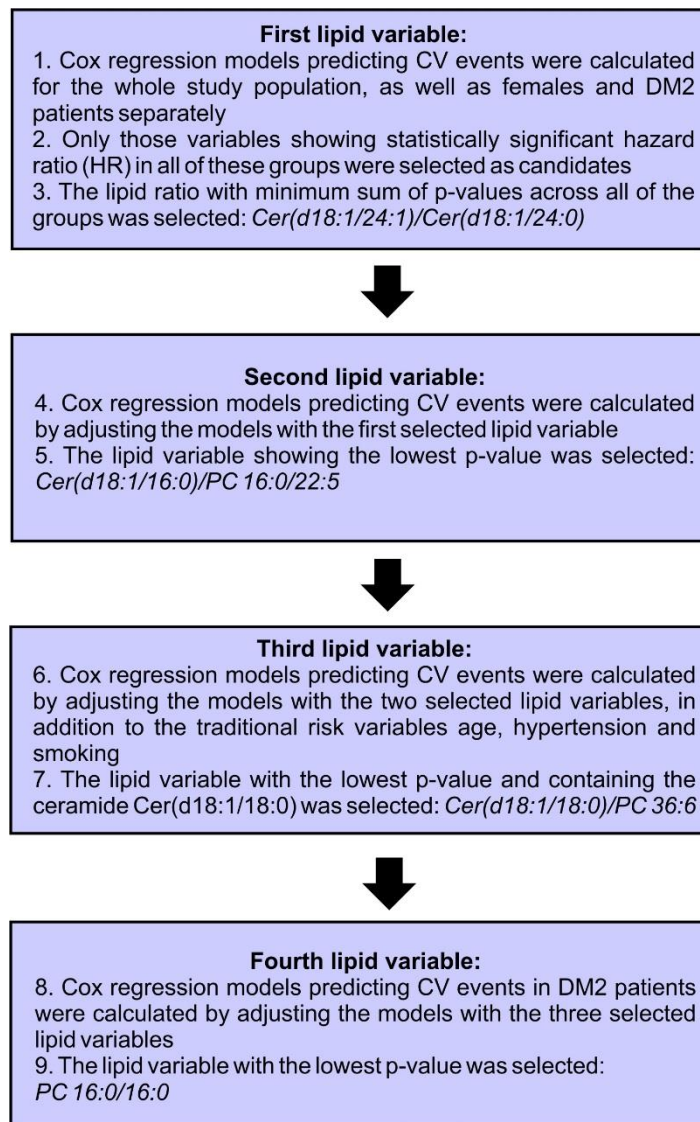
Variables	WECAC		KAROLA		LIPID	
	CV death	CV event	CV death	CV event	CV death	CV event
age + sex + CERT2	0.755	0.645	0.702	0.587	0.695	0.619
age + sex + systolic bp ^a + DM + current smoking	0.747	0.646	0.716	0.603	0.664	0.610
age + sex + systolic bp ^a + DM + current smoking + CERT2	0.769	0.661	0.745	0.620	0.700	0.628
age + sex + LDL-C	0.721	0.621	0.634	0.574	0.654	0.597
age + sex + LDL-C + CERT2	0.759	0.645	0.723	0.590	0.695	0.620
age + sex + hsCRP	0.724	0.624	0.627	0.569	-	-
age + sex + hsCRP + CERT2	0.755	0.645	0.703	0.589	-	-
age + sex + hsTnT	0.758	0.647	0.670	0.590	-	-
age + sex + hsTnT + CERT2 ^b	0.781	0.664	0.715	0.601	-	-
age + sex + NT-proBNP	-	-	0.674	0.600	-	-
age + sex + NT-proBNP + CERT2	-	-	0.718	0.609	-	-
age + sex + eGFR	0.727	0.621	-	-	0.676	0.612
age + sex + eGFR + CERT2	0.761	0.645	-	-	0.704	0.628
age + sex + HbA1c	0.723	0.620	-	-	-	-
age + sex + HbA1c + CERT2	0.757	0.645	-	-	-	-
age + sex + hsTnT + hsCRP + eGFR + LDL-C + HbA1c	0.764	0.648	-	-	-	-
age + sex + hsTnT + hsCRP + eGFR + LDL-C + HbA1c + CERT2	0.783	0.663	-	-	-	-

^ahypertension in KAROLA, ^bCERT2 and hsTnT used as continuous variables, i.e. not as CERT2-TnT score.

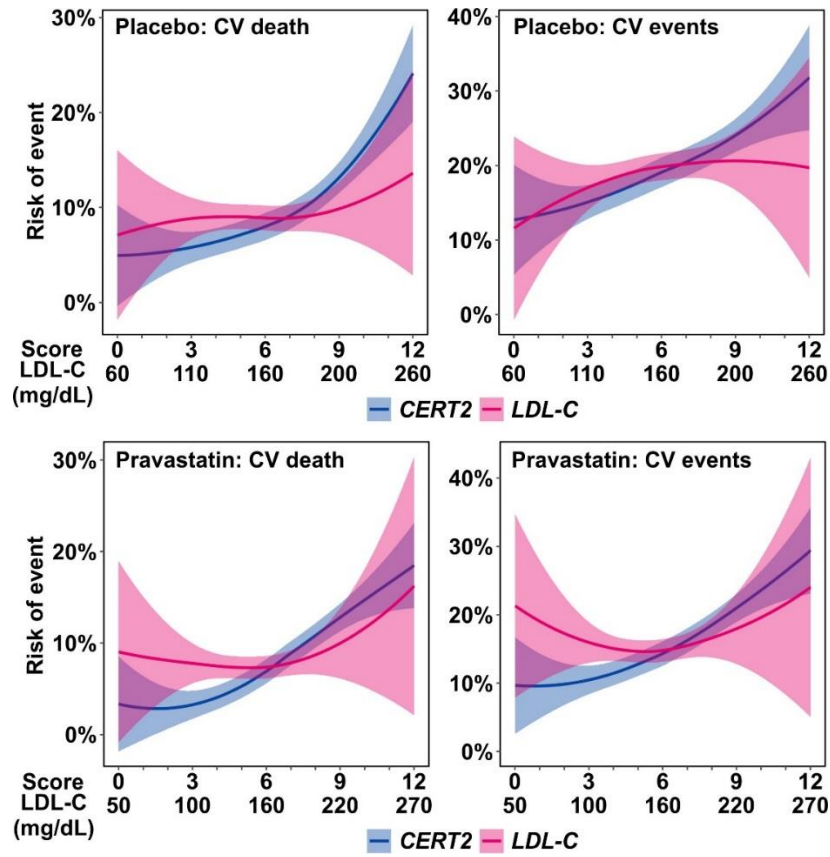
Supplementary Figures



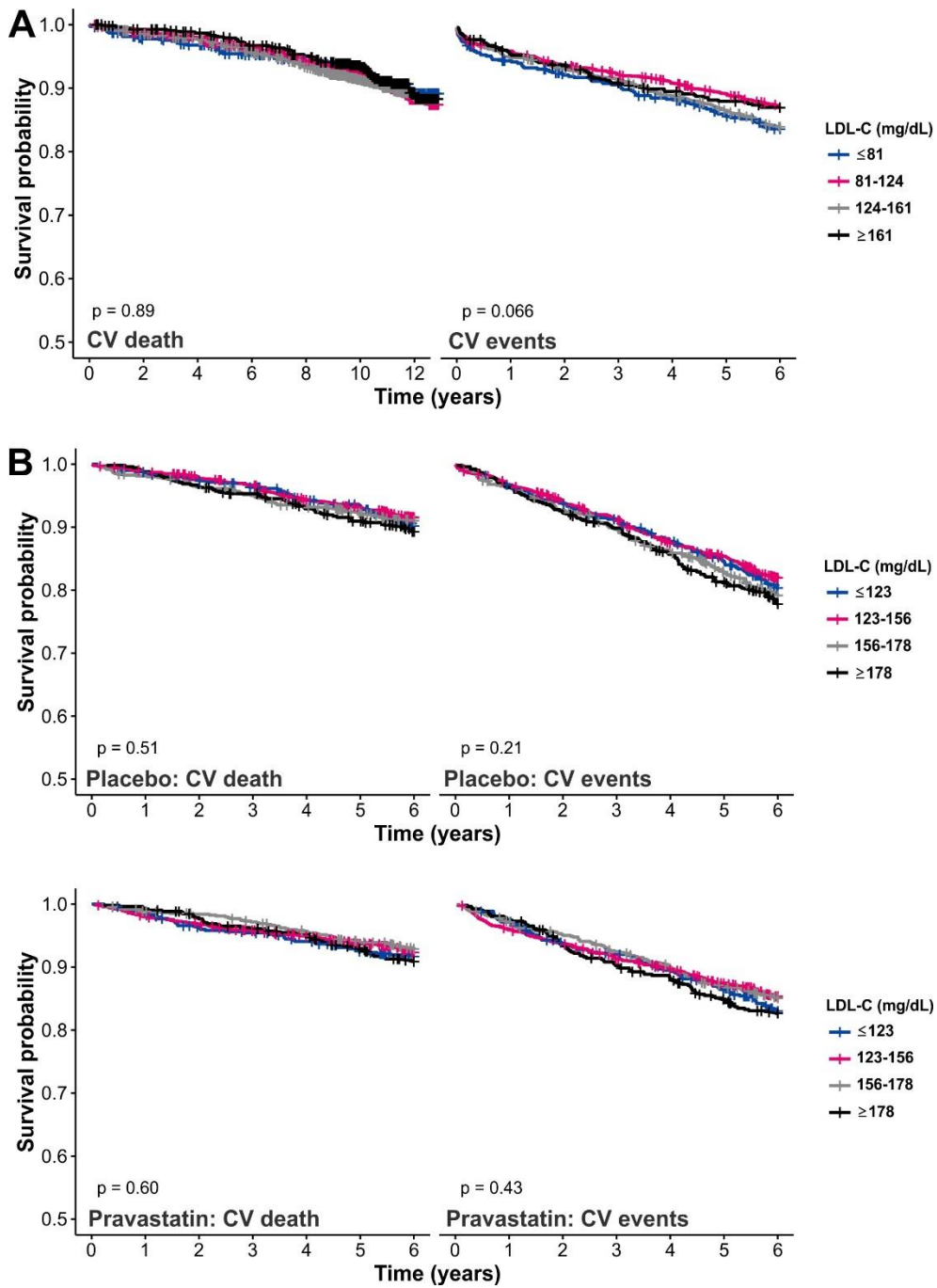
Supplementary Figure S1. Overlaid chromatograms for 1) screening and 2) LCPL analysis of an internal control sample with isotopically labelled PC standard lipids for the identification of A) PC 16:0/22:5(n-3), B) PC 14:0/22:6 C) PC 16:0/16:0 lipid species.



Supplementary Figure S2. Selection process of the lipid variables for the ceramide-phospholipid score (CERT2) that was developed based on the WECAC study data. For the selection, single ceramides and phospholipids, in addition to ceramide/ceramide and ceramide/phospholipid ratios were used. The aim was to develop a score of four components that would consist of lipid ratios or single lipids and to ensure that the score has at least one component that predicts risk also only in females, usually underrepresented in CV study cohorts, or subjects with diabetes mellitus type 2 (DM2). The selection of the score components was performed in a stepwise manner by constructing cox regression models and at each step adjusting the models with the previously selected lipids.



Supplementary Figure S3. Risk (6 years) curves for CERT2 and LDL-C in the LIPID trial placebo and pravastatin treatment arms separately.



Supplementary Figure S4. Kaplan Meier curves for LDL-C A) in the WECAC study and B) in the placebo and pravastatin treatment arms in the LIPID trial.