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Assessment of plasma ceramides as predictor for subclinical atherosclerosis

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ABSTRACT

Background and aims: Ceramides have been identified as novel biomarkers for cardiovascular disease (CVD) related events and mortality but their role in etiology of subclinical atherosclerosis is unknown. We aimed to assess association between plasma ceramides and carotid intima-media thickness (CIMT) and evaluate predictive value of the ceramides for high CIMT over traditional CVD risk factors.
Methods: Association between plasma ceramides and CIMT in the Young Finns Study participants was analyzed with CIMT as outcome and ceramides along with traditional risk factors as predictors with regression model. Predictive value of the ceramides and related coronary event risk test (CERT) score for high CIMT as surrogate marker of subclinical atherosclerosis was assessed by comparing logistic regression-based prediction models including, *i*) traditional risk factors and ceramides, *ii)* traditional risk factors and CERT score, *iii)* age, sex and ceramides or alternatively CERT score with a reference model including only traditional risk factors. The prediction models were fitted to training data (70% data) and tested on test data (30% data). The predictive models were assessed with area under the receiver operating curve (AUC). The variance of AUC was estimated by repeating the model fitting and testing for 1000 bootstraps of the original data.
Results: Predictive models with plasma ceramides or alternatively with CERT score in addition to age and sex variables were able to predict high CIMT with AUC 0.726 and 0.720 respectively. However, the ceramides and CERT score did not have statistically significant added predictive value for high CIMT over traditional risk factors.
Conclusions: The new systemic biomarkers, high-risk plasma ceramides and CERT score, showed promising predictive performance for high CIMT with only age and sex as additional variables. This may help in predicting subclinical atherosclerosis for primary prevention.

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of

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morbidity and mortality globally with major human and economic costs [1]. Atherosclerosis, the underlying pathology behind the majority of CVDs, begins decades before the clinical manifestations and silently reaches a stage where it can only be slowed down but not be reversed [2]. Therefore, timely identification of people at risk is crucial for prevention through lifestyle changes, dietary habits or drug interventions.

Numerous predictive methods have been developed for CVD risk stratification over the past two decades, such as the Framingham [3–5], SCORE [6], QRISK [7–9] and ceramide score [10]. Some of these methods are also in clinical practice, for example, the National Institute for Health and Care Excellence guidelines recommend using the Framingham method [12]. However, predictive methods designed for clinical CVD outcomes might not be able to detect subclinical changes which is crucial for primary prevention.

In-depth understanding of mechanisms underlying the development of atherosclerosis and identification of novel biomarkers are essential in developing or improving prediction methods for subclinical atherosclerosis. The traditional risk factors that are incorporated in most of the existing risk prediction algorithms for CVD have limited value in predicting subclinical atherosclerosis among low-risk population such as women and younger people. For example, a study by Ref. [20] found that even though traditional risk factors-based methods such as Ideal Cardiovascular Health Score (ICHS) and Fuster-BEWAT Score (FBS) predict subclinical atherosclerosis with similar accuracy, half of the study population with ideal ICHS and FBS scores had subclinical atherosclerosis. Therefore, identification and validation of novel biomarkers with additional or stand-alone predictive value for subclinical atherosclerosis is an important step forward in preventive cardiology.

In this study, we investigated the association of plasma ceramides with an early marker of atherosclerosis, i.e., carotid intima media thickness (CIMT) assessed with ultrasound imaging. We also assessed the plasma ceramides and a related score used for Coronary Event Risk Test (CERT) [10] for their predictive value for high CIMT as surrogate marker of subclinical atherosclerosis. Several studies have suggested that CIMT is an early and valid surrogate marker for CVD risk [26–28]. Ceramides are lipid molecules composed of sphingosine and a fatty acid that have recently been identified as a novel biomarker to predict adverse cardiovascular events including death [10,14,23,24]. We analyzed high-risk plasma ceramides, Cer(d18:1/16:0), Cer(d18:1/18:0) and Cer(d18:1/24:1) as well as ceramide, Cer(d18:1/24:0) which has been shown to be protective for CVD and has strong association with LDL-cholesterol [10,15]. The high-risk ceramides and their ratios with the protective ceramide have been shown to be useful in risk stratification for carotid artery disease patients [15]. However, to the best of our knowledge, the association of plasma ceramides with CIMT and their role in the prediction of high CIMT as a surrogate marker of subclinical atherosclerosis has not been studied yet. The high-risk ceramides as well as the protective ceramide were investigated for their ability in predicting subclinical atherosclerosis.

2. Materials and methods

2.1. Study subjects and ethics

The Cardiovascular Risk in Young Finns Study (YFS) is an ongoing follow-up study to access cardiovascular risk factors from childhood to adulthood. YFS is a prospective multi-center study initiated in 1980 with 3596 children and adolescents aged 3–18 years. The participants were randomly selected from areas of five university hospitals in Finland (Turku, Tampere, Helsinki, Kuopio and Oulu) and have been followed up for over 40 years. The present study is based on follow-up in 2007. CIMT measured in 2007 was used as proxy of subclinical atherosclerosis. An ultrasound imaging device with a high-resolution system (Sequoia 512, Acuson) was used for CIMT measurement by trained sonographers following a standardized protocol in the five centers. The measurements were taken for minimum of four times from posterior wall of the left common carotid artery ≈10 mm proximal to the carotid bifurcation. Traditional risk factors data was available from 2090 subjects (age:30–45 years, women:55%). Traditional risk factors, plasma ceramide and CIMT data are available for 2060 subjects (age:30–45 years, women:55%). The study complies with the Declaration of Helsinki, all participants gave written informed consent, and the study was approved by the local Ethics Committees (ETMK:68/1801/2017).

2.2. Traditional CVD risk factors

Traditional risk factors for CVD included in this study were age, sex, waist size, body mass index (BMI), systolic and diastolic blood pressure, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, insulin, glucose, C-reactive protein (CRP), smoking habit and family history as described in detail by Refs. [16,29].

2.3. Biochemical assays

Ceramides analyzed in this study were a subset of larger lipidome data quantified from stored plasma samples at Zora Biosciences Oy (Espoo, Finland). Lipid extraction was based on a previously described method [17]. In brief, 10 μl of 10 mM 2,6-di-tert-butyl-4-methylphenol (BHT) in methanol was added to 10 μl of the sample, 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). The samples were mixed and sonicated in a water bath for 10 min, followed by a 40-min incubation and centrifugation (15 min at 5700×*g*). 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 min. Then, 100 μl of methanol was added to the samples before the extracts were centrifuged for 5 min at 3500×*g*, and finally the supernatants were transferred to the analysis plate for mass spectrometric (MS) analysis. The MS analyses have also been described in detail in Ref. [18]. The analyses were performed on a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500, AB Sciex, Concord, Canada) equipped with ultra-high-performance liquid chromatography (UHPLC) (Nexera-X2, Shimadzu, Kyoto, Japan). Chromatographic separation of the lipidomic screening platform was performed on an Acquity BEH C18, 2.1 × 50 mm id. 1.7 μm column (Waters Corporation, Milford, MA, USA). The data were collected using a scheduled multiple reaction monitoring algorithm and processed using Analyst and MultiQuant 3.0 software (AB Sciex). The heights of the peaks obtained from the MS analysis were normalized with the internal standard of the lipid classes [33].

2.4. Statistical methods

Statistical analyses were performed using the R environment for statistical computing, version 3.6.1 [19]. Plasma ceramides analyzed in this study included high risk ceramides, Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:1) and protective ceramide, Cer(d18:1/24:0). We also analyzed association between CERT score, a score derived from these individual ceramides [10], and CIMT, followed by assessment of the predictive ability of CERT score for high CIMT. For the analyses, skewness in the values for waist circumference, BMI, total cholesterol, LDL cholesterol, HDL

cholesterol, triglycerides, insulin, glucose, CRP and ceramides were corrected with natural log transformation.

2.4.1. Association analysis

The association between CIMT (outcome) and traditional CVD risk factors (predictors) was assessed with stepwise multivariable linear regression modelling using backward selection implemented in *bootStepAIC* R package [11]. The backward variable selection algorithm starts with a model with all possible explanatory variables and then discards the least informative variables one by one based on Akaike information criterion (AIC) which was used in this study. AIC is a relative measure that quantifies the amount of information loss due to the elimination of a variable from a regression model. The potential sensitivity of stepwise regression to initial inputs was mitigated by repeatedly running stepwise regression on 100 bootstrap samples. Bootstrapping is a statistical technique that allows repeated use of the same dataset by sampling the dataset with replacement. This process is a well-validated and accepted statistical workaround for replicating an experiment multiple times with different samples from population which is often infeasible in biomedical research. The most frequently selected variables among the 100 stepwise regressions using the bootstrapped data were used as traditional CVD risk factors in our statistical reference model as described below.

2.4.2. Evaluation of prediction models

A reference prediction model (model 1) for high CIMT including only the selected traditional CVD risk factors as predictors was built. Added predictive value of the plasma ceramides and CERT score for high CIMT was accessed by comparing logistic regression-based prediction models including, *i*) traditional risk factors and ceramides (model 2), and *ii)* traditional risk factors and CERT score (model 3) with the reference prediction model. Similarly, predictive values of models with only ceramides or CERT score in addition to age and sex information were assessed by comparing prediction models with age, sex and ceramides (model 4) or alternatively the CERT score (model 5) with the reference model.

Dichotomization of the CIMT data was done by defining participants with CIMT value higher than or equal to 90th percentile as cases (with high CIMT) and those with CIMT value less than 90th percentile as controls. Assessment of the predictive value of the ceramides and the CERT score was performed using a standard train-test split machine learning approach. The approach involves splitting the original data into training and test subsets. The predictive models are trained using the training subset and tested on the test subset which serves as a proxy for new data. Specifically, in this study, the prediction model fitting and testing in the case-control setting was done by, i) fitting models to training data (70% data), ii) testing the models on test data (30% data), and iii) calculating area under the receiver operating curve (AUC) for predictive model assessment (sensitivity-specificity). The variance of AUC was estimated by repeating the model fitting and validation for 1000 bootstraps of the original data. Added predictive ability of the ceramides and CERT score over the traditional risk factors was tested by taking differences between AUCs (ΔAUC) obtained from model 1 and model 2 for ceramides and model 1 and model 3 for CERT score over 1000 bootstraps of the original data. The p-value was then estimated by counting the proportion of ΔAUC less than zero. The whole prediction model analysis was also repeated with CIMT data dichotomized using 75th percentile as threshold for high CIMT.

3. Results

3.1. Descriptive characteristics of the study population

A total of 2060 individuals had complete CIMT measurements with ultrasound, traditional risk factors, plasma ceramides and covariate data available. The characteristics of the study participants with complete data stratified by case-control are presented in Table 1.

3.2. Formation of predictive models for CIMT and selection of traditional risk factors

Stepwise multivariable linear regression using backward selection algorithm and 100 bootstraps was used to select the seven most significant risk factors out of 15 measured traditional risk factors as follows: age, sex, BMI, systolic and diastolic blood pressure, total cholesterol and HDL cholesterol. These seven risk factors were selected in at least 65% of the 100 bootstraps runs. The reference and other prediction models designed for CIMT prediction based on these selected traditional risk factors, ceramides and CERT are presented in the caption of Table 2.

3.3. Associations between CIMT and risk factors

Our results suggest that plasma ceramides are associated with CIMT when adjusted with age and sex variables [Table 2]. The association between the plasma ceramides and CIMT disappears after adjusting for other traditional risk factors considered in the study which suggests non independent association between the plasma ceramides and CIMT in the Young Finns Study cohort. The overall predictive ability of the reference model (model 1) for CIMT, as indicated by the coefficient of determination (R^2), is 20.9% [Table 2]. Similar results for predictive model with traditional risk factors from the same data has also been published elsewhere [16]. The predictive values of models 2 (R^2 = 21.1%) and 3 (R^2 = 21%) are mildly improved as compared to that of model 1. Models 4 and 5, designed to test predictive values of ceramides and CERT score only, in addition to age and sex variables, have lower predictive values (R^2 = 15.7% and 14.6%, respectively) as compared to that of model 1. However, the effect sizes (β) (strength of relationship with CIMT) and statistical significances (p-value) of the ceramides, Cer(d18:1/24:1) and Cer(d18:1/18:0) are increased in model 4 as compared to that of model 1 [Table 2]. Similarly, the effect size (β) and statistical significance (p-value) of the CERT score are increased in model 5 as compared to that of model 3 [Table 2].

3.4. Evaluation of prediction models with and without ceramides and CERT score

Mean and 95% confidence interval (CI) of AUCs obtained from prediction models with traditional risk factors only (model 1), plasma ceramides in addition to the traditional risk factors (model 2), CERT score in addition to the traditional risk factors (model 3), plasma ceramides with only age and sex variables (model 4) and CERT score with only age and sex variables (model 5) over 1000 bootstraps are presented in Table 3. Mean AUCs are slightly higher in models with plasma ceramides or CERT score in addition to the traditional risk factors (models 2 and 3) as compared to the reference model 1. However, the added predictive ability due to the plasma ceramides or CERT in the models 2 and 3 are not statistically significant [Fig. 1]. Assessment of the statistical significance was done by calculating the proportion of differences in the AUC (ΔAUC) less than zero obtained from predictive models for high CIMT using traditional risk factors without and with the addition of ceramides

Table 1
Characteristics of the study population.

	Cases	Controls	p-value
Number of subjects (%)	211 (10%)	1849 (90%)	–
Sex (female %)	64%	43%	–
Age, years	41(±4)	37(±5)	<2.2e-16
Traditional risk factors			
Waist circumference (cm)	96.3 (±14.4)	87.4 (±12.8)	<2.2e-16
Body mass index (kg/m^2)	28.3(±5.3)	25.7(±4.6)	4.3e-11
Systolic blood pressure (mmHg)	128 (±15)	120 (±14)	1.9e-13
Diastolic blood pressure (mmHg)	80 (±12)	75 (±11)	8.2e-09
Total cholesterol (mmol/l)	5.3 (±0.9)	5.0 (±0.9)	0.0001
LDL cholesterol (mmol/l)	3.3 (±0.8)	3.1 (±0.8)	4.9e-05
HDL cholesterol (mmol/l)	1.2 (±0.3)	1.4 (±0.3)	1.7e-08
Triglycerides (mmol/l)	1.6 (±0.8)	1.3 (±0.7)	6.7e-08
Serum glucose (mmol/l)	5.6 (±1)	5.3 (±0.8)	5.2e-05
Insulin (mU/l)	15.5 (±46.7)	8.5 (±7.7)	0.03
C-reactive protein (mg/l)	2.4 (±7.1)	1.8 (±3.1)	0.21
Daily smoking (%)	45 (21%)	333 (18%)	0.28
Carotid intima media thickness (CIMT) (mm)	0.82 (±0.07)	0.60 (±0.07)	<2.2e-16

Table 2
Associations between carotid intima media thickness (CIMT) and risk factors. Values are regression coefficients (β), expressed in micrometers, standard error of the regression coefficients (SE) and respective p-values.

Risk factor	Model 1 (reference)*		Model 2		Model 3		Model 4		Model 5	
	β ± SE	p-value	β ± SE	p-value	β ± SE	p-value	β ± SE	p-value	β ± SE	p-value
Age	29.4 ± 1.9	$<2 \times 10^{-16}$	29.4 ± 2	$<2 \times 10^{-16}$	29.4 ± 2	$<2 \times 10^{-16}$	31.3 ± 2	$<2 \times 10^{-16}$	32.2 ± 2	$<2 \times 10^{-16}$
Sex	11.1 ± 4.33	0.01	12.03 ± 4.4	0.007	11.6 ± 4.3	0.008	28.6 ± 4.1	5×10^{-12}	30.1 ± 3.4	8×10^{-14}
Body mass index	16.2 ± 2.2	3×10^{-13}	15.6 ± 2.3	2×10^{-11}	16.7 ± 2.2	7×10^{-14}	–	–	–	–
Systolic blood pressure	16.3 ± 3.09	1.4×10^{-7}	15.8 ± 3.1	4×10^{-7}	15.9 ± 3.1	3×10^{-7}	–	–	–	–
Diastolic blood pressure	−6.18 ± 3.05	0.04	−6.5 ± 3.07	0.03	−6.5 ± 3.1	0.03	–	–	–	–
Total cholesterol	4.4 ± 2.1	0.04	6.4 ± 2.6	0.01	4.7 ± 2.2	0.03	–	–	–	–
HDL cholesterol	−9.6 ± 2.2	2×10^{-5}	−9.6 ± 2.3	3×10^{-5}	−9.5 ± 2.3	3×10^{-5}	–	–	–	–
Cer(d18:1/24:1)	–	–	1.8 ± 3.4	0.6	–	–	3.5 ± 3.5	0.3	–	–
Cer(d18:1/16:0)	–	–	−6.1 ± 2.9	0.04	–	–	−7.3 ± 2.7	0.007	–	–
Cer(d18:1/18.0)	–	–	3.3 ± 2.9	0.26	–	–	14.9 ± 2.8	1×10^{-7}	–	–
Cer(d18:1/24:0)	–	–	−1.1 ± 2.6	0.68	–	–	−1.8 ± 2.6	0.49	–	–
CERT score	–	–	–	–	−0.13 ± 0.7	0.85	–	–	2.3 ± 0.66	0.0004
Coefficient of determination (R^2)	20.9%		21.1%		21%		15.7%		14.6%	

Abbreviation: BMI, body mass index; Cer, ceramide; CERT, coronary event risk test; SE, standard error.
Interpretation: β values are regression coefficients representing increment in CIMT in micrometers for a 1 SD change in continuous predictors variables and among males as compared to females.
Model 1*: *CIMT ~ Age + sex + BMI + systolic blood pressure + diastolic blood pressure + total cholesterol + HDL cholesterol.*
Model 2: CIMT ~ Age + sex + BMI + systolic blood pressure + diastolic blood pressure + total cholesterol + HDL cholesterol + Cer(d18:1/16:0) + Cer(d18:1/18:0) + Cer(d18:1/24:0) + Cer(d18:1/24:1).
Model 3: CIMT ~ Age + sex + BMI + systolic blood pressure + diastolic blood pressure + total cholesterol + HDL cholesterol + CERT.
Model 4: CIMT ~ Age + sex + Cer(d18:1/16:0) + Cer(d18:1/18:0) + Cer(d18:1/24:0) + Cer(d18:1/24:1).
Model 5: CIMT ~ Age + sex + CERT.

Table 3
Area under receiver operating characteristic curve (AUC) parameters (mean, 95% Confidence Interval (CI)) of analyzed predictive models over 1000 bootstraps.

Risk factors	Mean AUC	95% CI
*Results based on dichotomization of the CIMT data using 90*th *percentile threshold (0.75* mm*, CIMT > 90*th *percentile was considered high)*		
Traditional risk factors (Model 1)	0.757	[0.696, 0.811]
Traditional risk factors + ceramides (Model 2)	0.762	[0.697, 0.818]
Traditional risk factors + CERT (Model 3)	0.760	[0.696, 0.819]
Age + sex + Ceramides (Model 4)	0.726	[0.661, 0.784]
Age + sex + CERT (Model 5)	0.720	[0.660, 0.778]
*Results based on dichotomization of the CIMT data using 75*th *percentile threshold (0.68* mm*, CIMT > 75*th *percentile was considered high)*		
Traditional risk factors (Model 1)	0.722	[0.675, 0.766]
Traditional risk factors + ceramides (Model 2)	0.729	[0.688, 0.771]
Traditional risk factors + CERT (Model 3)	0.734	[0.690, 0.776]
Age + sex + Ceramides (Model 4)	0.710	[0.666, 0.754]
Age + sex + CERT (Model 5)	0.705	[0.663, 0.749]

Abbreviations: CERT, coronary event risk test.

or alternatively coronary event risk test (CERT) score over 1000 bootstraps. Models 4 and 5, however, indicate that plasma ceramides and CERT score might be potential stand-alone predictor of subclinical atherosclerosis in absence of traditional risk factors other than age and sex variables.

4. Discussion

In this study, we, for the first time, present an assessment of association between plasma ceramides and CIMT and evaluate their predictive ability for high CIMT as surrogate marker of subclinical atherosclerosis. Our results show that plasma ceramides, which have been shown to predict major cardiovascular events are also associated with CIMT when only age and sex variables are used as covariates. This could be due to ceramide's ability to express risks on behalf of the other risk factors involved in atherosclerotic pathogenesis since the association between plasma ceramides and

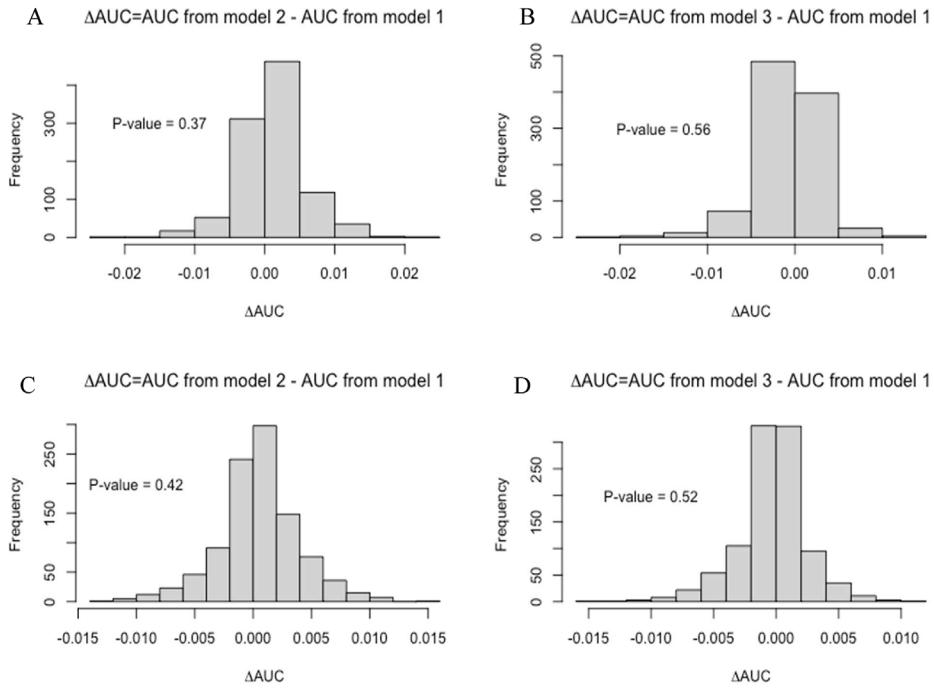

Fig. 1. Distribution of differences in the area under the receiver operating curve (ΔAUC) obtained from predictive models for carotid intima media thickness using traditional risk factors without and with the addition of ceramides or alternatively coronary event risk test (CERT) score over 1000 bootstraps. The comparison was done twice using CIMT data dichotomized using 90th percentile, where CIMT >90th percentile was considered high (panels A and B) and 75th percentile, where CIMT >75th percentile was considered high (panels C and D). P-values are shown too. (Model 1: *CIMT ~ Age + sex + BMI + systolic blood pressure + diastolic blood pressure + total cholesterol + HDL cholesterol.* Model 2: CIMT *~Age + sex + BMI + systolic blood pressure + diastolic blood pressure + total cholesterol + HDL cholesterol + Cer(d18:1/16:0) + Cer(d18:1/18:0) + Cer(d18:1/24:0) + Cer(d18:1/24:1).* Model 3: CIMT~ *Age + sex + BMI + systolic blood pressure + diastolic blood pressure + total cholesterol + HDL cholesterol + CERT score.).*

CIMT disappears after adjusting with other traditional CVD risk factors. The results indicate that the plasma ceramides might have stand-alone predictive ability for high CIMT when used only with age and sex variables and therefore might be a potential biomarker for subclinical atherosclerosis.

We found that, out of the four studied plasma ceramides, *Cer(d18:1/16:0)* and *Cer(d18:1/18:0)* are significantly associated with CIMT after adjusting only for age and sex. The association between the ceramides and high CIMT disappears after adjusting for all the major traditional risk factors used in this study, perhaps due to the excess ceramide related variance in CIMT already being expressed through the traditional risk factors such as total cholesterol that are moderately correlated with the ceramides. Similarly, the predictive ability of the plasma ceramides for subclinical atherosclerosis, when used only with age and sex variables, could be due to ceramide's ability to express risks on behalf of the other risk factors involved in atherosclerotic pathogenesis.

Plasma ceramides are already in use in Mayo Clinic Laboratories to predict adverse cardiovascular events (Mayo Clinic: https://news.mayocliniclabs.com/ceramides-miheart/). While prediction of clinical CVD events is crucial for risk stratification and patient management [10,23,24], ability for early assessment by identifying subclinical changes is important for primary prevention. This requires identification of novel predictor biomarkers in addition to the traditional risk factors as people with subclinical atherosclerosis might have optimal traditional risk factors profile [20]. Our results indicate that plasma ceramides might predict CVD in subclinical phase as well and highlight their potential importance in primary prevention of CVD. The effect sizes of the ceramide *Cer(d18:1/18:0)* as well as CERT score were higher in the models 4 and 5 as compared to those in the reference model 1 [Section 3.3]. We speculate that the increased effect sizes suggest that the ceramides and CERT score represent combined effects across traditional risk factors. This finding, if validated with additional studies, might have an important role in developing efficient screening test for CVD.

Replacement of several traditional risk factors with fewer biomarkers such as ceramides or CERT score can help to minimize errors accumulated across multiple measurements [25,32]. For example, prediction of high CIMT has been shown to be improved after replacing traditional risk factors such as total cholesterol and HDL cholesterol with nuclear magnetic resonance (NMR)-determined LDL cholesterol, medium size HDL, docosahexaenoic acid, and tyrosine [30]. Similarly, adiponectin levels improve prediction of high CIMT in adults over traditional risk factors [31].

There are some limitations of this study. The number of study participants with high CIMT (≥90th percentile) were only 211 (~10%). CIMT was used as a surrogate marker for subclinical atherosclerosis due to lack of artery plaque measurement data in sufficient number of YFS cohort participants. For practical reasons, this study is based on intima media thickness measurements of a single segment, that is the posterior wall of the left common carotid artery ≈10 mm proximal to the carotid bifurcation. This lack of scanning of other artery segments and branches may result in loss of information and patient misclassification. The study is based on geographically constrained population and lacks replication of the results in an independent cohort.

Even though measuring plasma ceramides is considered comparatively expensive, the benefits outweigh the cost as the molecules provide simple biomarkers that can potentially be used for screening early CVD risk among asymptomatic individuals with addition of only age and sex information. This benefit avoids the need to collect large number of traditional predictive risk factors that may not be easily available and are time-consuming. A single

blood-based test is relatively more convenient to perform and provides systemic risk of atherosclerosis in whole vascular tree. Some biomarkers can have optimal profile among individuals with subclinical atherosclerosis [20]. Identification and addition of new potential biomarkers such as plasma ceramides in the existing list of predictors in literature might allow development and improvement of prediction methods with an optimal combination of the predictors.

In conclusion, the new systemic biomarkers, high risk ceramides and CERT score, showed promising predictive performance for high CIMT with only age and sex as additional variables. The biomarkers could potentially be useful for screening early CVD risk among asymptomatic individuals for primary prevention.

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CRediT authorship contribution statement

Pashupati P. Mishra: Conceptualization, Investigation, Writing - original draft. **Binisha H. Mishra:** Investigation, Writing - review & editing. **Leo-Pekka Lyytikäinen:** Writing - review & editing. **Mika Hilvo:** Data analysis, Writing - review & editing. **Markus Juonala:** Writing - review & editing. **Mika Kähönen:** Writing - review & editing. **Nina Hutri-Kähönen:** Writing - review & editing. **Dimitrios I. Fotiadis:** Writing - review & editing, Funding acquisition. **Olli T. Raitakari:** Writing - review & editing. **Reijo Laaksonen:** Data acquisition, Writing - review & editing. **Terho Lehtimäki:** Supervision, Conceptualization, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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