Ceramides versus standard methods in prediction of subclinical atherosclerosis

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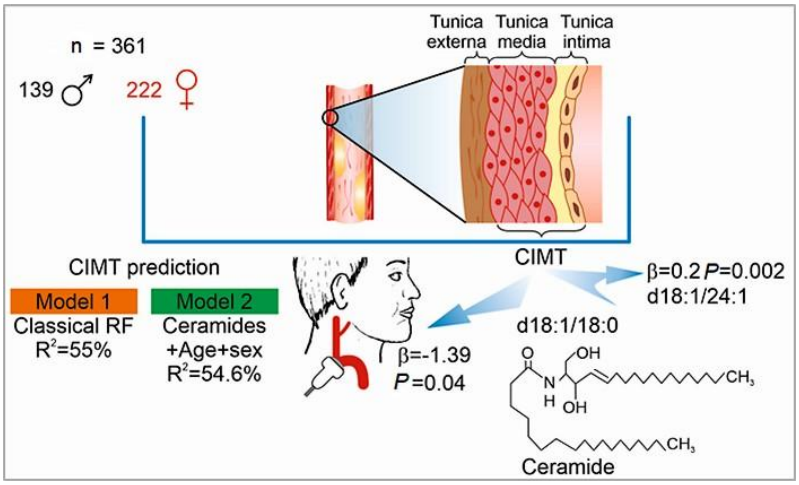
Background. Ceramides are a heterogeneous group of bioactive membrane sphingolipids that play specialized regulatory roles in cellular metabolism depending on their characteristic fatty acyl chain lengths and subcellular distribution. Ceramides have emerged as promising biomarkers for cardiovascular diseases.
Methods. Within the prospective Kardiovize study based in Brno, Czech Republic, we aimed to develop two carotid intima–media thickness (CIMT) prediction models using available variables. One model incorporated, in addition to sex and age, conventional biomarkers (total cholesterol, triglycerides, lipid-lowering therapy), while the other included blood concentrations of selected ceramides. Lipids species were measured by a hyphenated mass spectrometry technique.
Results. A total of 139 men (mean age 61.8 ± 16.4) and 222 women (63.9 ± 14.8) were included in the present study. Both approaches yielded almost identical coefficients of determination (54.6 % vs 55.0%) with good classification abilities (weighted kappa 0.69 vs 0.71) in both models. The ceramide (d18:1/18:0) shows a strong negative association with CIMT (β = −1.39, *P*=0.04), while (d18:1/24:1) shows a positive association with carotid intima–media thickness (β=0.20, *P*=0.002).
Conclusion. The tested ceramides were able to predict subclinical atherosclerosis by carotid intima–media thickness with comparable accuracy to a combination of routinely tested lipids.

Ceramides Versus Standard Methods In Prediction Of Subclinical Atherosclerosis



Plasma ceramides predicted subclinical atherosclerosis, as reflected by CIMT, with accuracy comparable to conventional lipid-based models.

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Graphical Abstract
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INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death globally, with an estimated 19.8 million lives each year[1]. Traditional cardiovascular risk factors (e.g. hypertension, diabetes, high low-density lipoprotein, smoking, overweight and obesity) explain only 75% of the risk for CVD and exposure to one or more vascular risk factors is common even in individuals who will not develop any clinical manifestations[2]. Most cardiovascular events are caused by atherosclerosis, which develops decades before clinical signs and insidiously progresses to a point where it can only be delayed but not reversed[3,4]. Therefore, it is essential to identify individuals at risk to prevent the onset of CVD with lifestyle interventions or medical treatment[5].

Carotid intima–media thickness (CIMT) measurement assesses the health and integrity of the carotid arteries, the major blood vessels supplying oxygenated blood to the brain. CIMT can detect subclinical atherosclerosis, characterized by the early formation of fatty deposits in the arteries. Several studies have suggested that CIMT is an early, valid and independent surrogate marker for CVD risk[6,7]. However, it should be noted that CIMT is primarily used as a marker of subclinical atherosclerosis in epidemiological and research settings and does not directly quantify the burden of atherosclerotic plaque. While CIMT can facilitate reclassification of patients at intermediate cardiovascular risk, its utility for individual risk stratification in routine clinical practice is limited and should be considered within a broader context of established cardiovascular risk assessment tools. Existing evidence suggests that a combination of demographic and clinical data, along with both conventional and novel biomarkers like ceramides, could effectively predict CIMT. This strategy holds considerable promise for improving clinical decision-making and public health outcomes[8]. However, the coefficients of determination (R^2) values are still relatively modest (0.21 to 0.45), and models have only been tested on primarily young and healthy populations[9,10].

Ceramides are a heterogeneous group of bioactive membrane sphingolipids that play specialized regulatory roles in cellular metabolism depending on their characteristic fatty acyl chain lengths and subcellular distribution[11]. They have an important role in many cellular functions such as apoptosis and inflammation[12]. During the past decade emerging clinical data have shown that ceramides also have a diagnostic value. Based on abundant published data, risk scores using the concentrations of circulating ceramides have been developed and adapted for routine clinical practice. Currently serum ceramides are used clinically as efficient risk stratifiers for primary and secondary prevention of atherosclerotic CVD such as coronary artery disease, stroke, as well as heart failure and atrial fibrillation[11,13-19]. To bring new insights into this field, the objective was to create two predictive models for CIMT using easily obtainable variables. One model is based on traditional biomarkers, such as blood lipid levels, while the other includes newer biomarkers, specifically ceramides.

METHODS

Study population and design

The Kardiovize study is a multidisciplinary epidemiological project with a random stratified by sex sample of 25–64-year-old residents of the city of Brno in Czechia designed as a prospective cohort[20,21]. The recruitment and core baseline examinations started on 31.01.2013. With a few additional measurements including cognitive tests, they were completed on 09.01.2015. Follow-up will be carried out regularly at 5-year intervals, with the first follow up starting 17.10.2017 and finalized by 21.03.2019. This analysis used an existing dataset; therefore, the sample size was determined by the number of eligible participants with CIMT and ceramide data available during the study period (n=361). No a priori power calculation was performed because participants were not prospectively enrolled to achieve a pre-specified effect size. The final multivariable models were intentionally parsimonious, and the available analytic sample provided a favorable observations-to-parameters ratio (Model 1, n=314; Model 2, n=315), supporting stable estimation and minimizing overfitting. We report coefficients with standard errors and p-values to reflect estimation precision. The Kardiovize study was approved by the Ethics Committee of the St Anne's University Hospital, Brno, Czechia. The study protocol complied with the latest Helsinki declaration and all participants signed the informed consent.

Data collection and measurement

Blood pressure was self-measured by the patients using an automated office measurement device (BpTRU, model BPM 200; Bp TRU Medical Devices Ltd., Coquitlam, BC, Canada). Anthropometric assessments included height, weight, body mass index (BMI), and waist circumference. Data on the use of drugs (lipid-lowering therapy, antihypertensives) were also collected, as well as basic biochemical parameters (high-density cholesterol, HDL, low-density cholesterol, LDL, total cholesterol, triacylglycerols). Laboratory tests were performed from 12-hour fasting whole blood samples using a Modular SWA P800 analyser (Roche, Basel, Switzerland) or enzymatic colorimetric methods (RocheDiagnostics GmbH, Hamburg, Germany), all evaluated at St Anne's University Hospital by trained research nurses.

Lipid extraction and LC-ESI/MS analysis

Serum blood samples were stored at −80 °C until lipidomic analysis. Serum lipids were extracted according to Folch, Lees, and Sloane Stanley (1957). Briefly, 100 µL of serum was mixed with chloroform/methanol (2:1, v/v) and $CaCl_2$ (0.025%, w/v) containing a deuterated internal lipid standard (Equisplash, Avanti Polar Lipids, Alabaster, AL, USA) and shaken using a thermoshaker (TS -100, Biosan, Riga, Latvia) at 1300 rpm. The samples were sonicated for 30 s (Kraintek 18, Hradec Kralove, Czech Republic). After centrifugation (16,000 × g, 15 min, 4 °C; Eppendorf 5427R, Eppendorf, Germany), the bottom phase was withdrawn and the upper layer was processed again using the same Folch extraction.

The organic phases were combined, evaporated using a rapid vacuum, and subsequently reconstituted in acetonitrile:water (3:1, v/v). Lipid extracts were analysed using an HPLC system consisting of a Thermo Scientific Dionex UltiMateTM 3000 RSLCnano system connected to an ABSciex QTRAP 6500 system as previously described[22]. A Waters Acquity UPLC BEH HILIC, 1.7 µm, 2.1 × 100 mm (Waters, Milford, MA, USA) column with a precolumn, Acquity UPLC BEH HILIC 1.7 µm VanGuard Pre-Column 2.1 × 5 mm (Waters, Milford, MA, USA), was eluted at a flow rate of 400 µL/min in a linear gradient from 0 to 10 min on 80% mobile phase A (water/acetonitrile [5:95, v/v] with 10 mM ammonium acetate) and over 11 min on 98% mobile phase B (water/acetonitrile [50:50, v/v] with 10 mM ammonium acetate; pH = 8.2). Mass spectrometry analyses were performed with electrospray ionisation in positive/negative mode and MRM scan, with a resolution of 0.7 +/− 0.1 µm (ref.[23]). MS additional parameters were as follows: curtain gas 35; temperature 500 °C; Ion Source Gas 1 40; Ion Source Gas 2 50; Ion spray voltage 5200. A library of theoretical precursor ions was generated for ceramides, with the sphingosine of d18:1 and varying the length of the fatty acid fraction. The characterization of ceramides species was achieved in positive mode with the formation of $[M+H]^+$. The collision energy was 43 eV. Data analysis was carried out using the Skyline daily 4.2.1.19058 software (MacCoss Lab, Department of Genome Sciences, University of Washington, Seattle, WA, USA).

Assessment of vascular structure using carotid intima–media thickness

Two trained investigators performed carotid ultrasonography to assess the CIMT. Before the study, the reliability of the recordings was evaluated using the intra-class correlation coefficient, which showed values of 0.97 (95% CI:0.94 to 0.99) for intra-observer agreement of repeated measurements in 20 subjects, and 0.90 (95% CI:0.74 to 0.96) for interobserver agreement. A Sonosite Micromax ultrasound (SonoSite, USA) device paired with a 5–10 MHz multifrequency high-resolution linear transducer with Sonocal software was used to automatically measure the CIMT. The measurements were performed on patients lying in a supine position with their heads extended and slightly turned opposite to the carotid artery examined. 120 CIMT values were obtained automatically in each patient along the common carotid artery, examining a 10-mm long part starting at a distance of 1 cm from the bifurcation.

Statistical analysis

All regression analyses were performed using complete-case data; participants with missing values in any of the covariates or outcome variables were excluded from the corresponding analyses. The proportion of missing data for key variables was minimal and did not materially affect the available sample size. Missing data were handled using a complete-case approach (listwise deletion) for each analysis. Participants with missing CIMT (imt_avg) or missing values in any covariate included in a given model were excluded from that model. No imputation was performed. In the analysis of the study population's characteristics, descriptive statistics are presented with means ± standard deviation for continuous variables. Comparative analysis between men and women was conducted using t-tests for continuous variables and the chi-square test for categorical variables. Variable selection was performed using backward elimination. The procedure began with a full model including prespecified candidate covariates; at each step, the covariate with the highest p-value was removed if it exceeded the predefined removal threshold (P>0.10). Models were refit iteratively until all remaining covariates satisfied the retention criterion (P≤0.10). Age and sex were forced into all models a priori. No interaction terms were included. Waist circumference, BMI, use of lipid-lowering therapy, and systolic and diastolic blood pressures were all included in both models. Model 1 also included HDL, LDL, triglycerides, and total cholesterol; and Model 2 included high-risk plasma ceramides[24-26], namely: Ceramide (d18:1/24:1), Ceramide (d18:1/16:0), Cerd18:1/18:0, and Ceramide (d18:1/24:0). By repeatedly running the regression on 100 bootstrap samples, the validity of both stepwise regressions was verified. Candidate covariates were selected a priori based on biological plausibility and prior literature and included demographic factors (age, sex), anthropometric indices (BMI and waist circumference), blood pressure variables, lipid-lowering therapy, routinely measured lipid parameters, and ceramides. Because BMI and waist circumference capture overlapping aspects of adiposity and may be collinear, we assessed collinearity during model specification (BMI and waist circumference were strongly correlated; r≈0.86, and VIF values were in the ~5–6 range when entered jointly). Therefore, BMI and waist circumference were treated as alternative adiposity indicators during covariate screening and were not forced simultaneously into the final model specification, to maintain interpretability and parsimony.

Additional established cardiovascular risk factors (e.g., smoking status, diabetes mellitus, antihypertensive treatment) were considered; however, these variables were not consistently available/complete in the analytic dataset and would have reduced the effective sample size and increased the risk of overfitting relative to the number of parameters. Accordingly, the final models focused on a parsimonious set of predictors aligned with the study

objective (lipid- and ceramide-related predictors), while retaining core demographic and vascular covariates available across participants.

Although most continuous variables did not strictly follow a Gaussian distribution (as documented in the Results section), visual inspection of residual plots from the final regression models indicated that the assumptions of linear regression were reasonably satisfied. In combination with the use of bootstrapping (100 resamples), which provides robust coefficient estimation without strict reliance on normality assumptions, this analytical approach was appropriate and robust for estimating the associations between predictors and CIMT. Because the lipid-based and ceramide-based models are non-nested, likelihood-ratio testing was not used for direct comparison. Instead, models were compared using AIC and BIC (lower values indicate better penalized fit). For fair comparison, both models were additionally refit on the common complete-case sample across all covariates included in either model, and RMSE and adjusted R^2 were also reported.

The calculation of quadratic weighted Cohen's kappa was used to determine the reliability of the models. The test evaluated the degree of agreement between the measured and model-estimated quartiles. Perfect agreement is implied by a coefficient value of 1.0. Higher the value, more accurate the model. The weighted kappa is a variation of the kappa coefficient that takes into account the degree of disagreement between categories. It is used when the categories being rated have an ordinal or hierarchical structure. CIMT values were categorized into quartiles based on the empirical distribution of CIMT measurements in the study sample (n=361). This distribution-based approach ensured that each quartile contained approximately equal numbers of participants. All statistical analyses were performed in the software Stata Standard Edition for Windows, version 14.

RESULTS

Subject characteristics

A total of 361 patients were included in the analysis. The mean age was 63.1 ± 15.5 years, 38.5% were men. Table 1 shows the differences in the basic demographic and physiological parameters between the sexes. Significant differences between sexes were detected in the diastolic blood pressure, body weight, height, and waist circumference. Other factors, such as age, BMI, lipid profiles, ceramide levels, and lipid-lowering therapy usage, did not show significant differences. Continuous variables except for CIMT did not have Gaussian distribution.

Associations between CIMT and ceramides

Table 2 compares the two models, highlighting the impact of various variables on CIMT. Both models demonstrate that sex and age are significant predictors of CIMT (P<0.001). The positive β values for sex and age indicate that male sex and older age were associated with increased CIMT. In Model 1, total cholesterol showed a positive relationship with CIMT (β=0.014, P=0.01). Triglycerides showed a negative relationship with CIMT (β= −0.027, P=0.002). The use of lipid-lowering therapy was an important variable in both models, although it reached significance only in Model 1 (0.04 in Model 1 vs. 0.06 in Model 2).

To compare model fit while accounting for complexity, both models were refit on a common complete-case sample (n=314) and information criteria were computed. Model 1 showed lower AIC and BIC than Model 2 (AIC −550.26 vs −545.06; BIC −524.02 vs −511.31), while RMSE was similar (0.0995 vs 0.1000) and adjusted R^2 was slightly higher for Model 1 (0.538 vs 0.533).

In Model 2, Ceramide (d18:1/16:0) was not statistically significant (P=0.13). Notably, the Ceramide

Table 1. Characteristics of the study population.

	Men (n = 139)	Women (n = 222)	*P*
Age, years, mean (SD)	61.8 ± 16.4	63.9 ± 14.8	0.20
Systolic blood pressure, mm Hg	127.7 ± 17.9	128.8 ± 21.2	0.60
Diastolic blood pressure, mm Hg	79.0 ± 9.0	76.4 ± 9.7	0.01
Body weight, kg	85.1 ± 13.3	72.6 ± 14.0	<0.001
Height, cm	177.5 ± 7.6	164.0 ± 7.0	<0.001
BMI	27.0 ± 3.8	27.0 ± 5.0	0.94
Waist circumference, cm	99.0 ± 11.6	91.6 ± 13.7	<0.001
Total cholesterol, mmol/L	5.1 ± 1.0	5.2 ± 1.1	0.57
Triglycerides, mmol/L	1.2 ± 0.5	1.2 ± 0.7	0.91
HDL-C, mmol/L	1.5 ± 0.3	1.5 ± 0.4	0.13
LDL-C, mmol/L	3.1 ± 0.8	3.1 ± 0.9	0.80
Ceramide (d18:1/16:0), μmol/L	0.02 ± 0.05	0.02 ± 0.05	0.45
Ceramide (d18:1/18:0), μmol/L	0.01 ± 0.03	0.01 ± 0.04	0.86
Ceramide (d18:1/24:0), μmol/L	0.04 ± 0.13	0.03 ± 0.13	0.48
Ceramide (d18:1/24:1), μmol/L	0.14 ± 0.46	0.14 ± 0.50	0.95
Lipid-lowering therapy, %	4.3	1.8	0.16
CIMT, mm	0.70 ± 0.17	0.67 ± 0.13	0.07

BMI, body mass index; CIMT, carotid intima–media thickness; HDL-C, high-density cholesterol; LDL-C, low-density cholesterol.

Table 2. Predictive models of carotid intima–media thickness.

	Model 1		Model 2	
Variables	β ± SE	*P*	β ± SE	*P*
Sex	0.04 ± 0.01	0.001	0.04 ± 0.01	<0.001
Age	0.006 ± 0.0004	<0.001	0.006 ± 0 .0004	<0.001
Total cholesterol	0.014 ± 0.005	0.01	–	–
Triglycerides	−0.027 ± 0.009	0.002	–	–
Lipid-lowering therapy	0.07 ± 0.04	0.04	0.07 ± 0.04	0.06
Ceramide (d18:1/16:0)	–	–	0.43 ± 0.28	0.13
Ceramide (d18:1/18:0)	–	–	−1.39 ± 0.67	0.04
Ceramide (d18:1/24:0)	–	–	−0.47 ± 0.19	0.01
Ceramide (d18:1/24:1)	–	–	0.20 ± 0.06	0.002
Intercept	0.22 ± 0 .03	<0.001	0.26 ± 0.02	<0.001
R^2	55.0%		54.6	

B, regression coefficients; R^2, coefficient of determination; SE, standard error.

Table 3. Quadratic weighted kappa results for the comparison between estimated and achieved quartiles.

Comparisons	Agreement	Expected agreement	Weighted kappa	Classification
Model 1	91.3 %	71.3 %	0.69	Good
Model 2	92.1 %	73.0 %	0.71	Good

CIMT Quartiles: Q1 < 0.58, Q2 0.58–<0.68, Q3 0.68–<0.77, and Q4 ≥ 0.77

(d18:1/18:0) showed a strong negative association with CIMT (β= −1.39, *P*=0.04), while Ceramide (d18:1/24:1) showed a positive association (β=0.20, *P*=0.002). Both models yielded significant intercepts and demonstrated very similar explanatory power, with Model 1 accounting for 55.0% of the variability in CIMT and Model 2 accounting for 54.6%. Similarly, the weighted kappa values demonstrated comparable classification performance between models (0.69 vs. 0.71), indicating equivalent overall predictive capability.

The ceramide Cerd18:1/18:0 showed a strong negative association with CIMT while ceramide Cerd18:1/24:1 showed a positive association with CIMT. Tested ceramides were able to predict subclinical atherosclerosis by CIMT with comparable accuracy to a combination of routinely tested lipids. Both Models demonstrated high accuracy in predicting quartile classifications (Table 3), as evidenced by the high agreement percentages and substantial quadratic weighted kappa scores, suggesting their effective predictive capabilities. Model 2 shows a slightly higher agreement and a higher weighted kappa score compared to Model 1, indicating it may be a more accurate model for estimating quartiles. The "Good" classification for both models indicates strong reliability in their estimations, making them suitable for practical applications where estimating quartiles is important.

DISCUSSION

In our study, the ceramide-based model achieved explanatory power and quartile-classification performance comparable to the conventional lipid-based model, as reflected by similar coefficients of determination (R^2 54.6 vs. 55.0) and quadratic weighted kappa values (0.71 vs. 0.69). This finding supports the concept that circulating ceramides may serve as complementary biomarkers associated with subclinical atherosclerosis as captured by CIMT. However, ceramide profiling relies on specialized lipidomic platforms, is associated with higher analytical costs, and is not widely available in routine clinical laboratories. Although ceramide panels are available in selected reference laboratories and have been implemented in some healthcare settings, broader uptake will depend on demonstrating clear incremental benefit beyond standard lipids and established clinical risk tools. Therefore, at present, ceramide measurements are most appropriately considered for selected patient populations or research contexts where advanced lipid stratification may be clinically warranted, rather than as a replacement for standard lipid panels.

The incremental clinical value of ceramide measurements will depend on whether they improve risk characterization beyond standard lipids and established multivariable risk tools, and whether such gains justify the additional cost and complexity. In the present analysis, the ceramide-based model did not materially outperform the conventional lipid-based model (R^2 and quadratic weighted kappa were very similar), which suggests that routine replacement of standard lipid testing is not supported by these data. A more realistic near-term role is as a complementary test in selected scenarios where conventional lipids may be insufficient or discordant with the clinical phenotype; for example, individuals with premature or progressive atherosclerotic disease despite 'normal' standard lipid profiles, patients in secondary/tertiary prevention where residual risk remains high, or research settings requiring refined lipid stratification. Importantly, demonstrating incremental utility requires formal evaluation using established performance metrics (e.g., changes

in discrimination, calibration, and reclassification) and, ideally, decision-analytic approaches (e.g., net benefit) and cost-effectiveness analyses. Accordingly, future longitudinal studies should test whether adding ceramides to conventional risk models meaningfully improves prediction of clinically relevant outcomes, and whether such improvements are feasible and economically justified in real-world practice.

The differential β coefficients for various ceramides underscore the complexity of their relationship with CIMT. Ceramides, sphingolipids central to the cell membrane integrity and signalling, have emerged as key players in CVD, with specific ceramide ratios being predictive of adverse cardiovascular events[14,27]. The findings of this study align with this emerging narrative, suggesting that not all ceramides are equal in their cardiovascular implications and that a detailed lipidomic profile could enrich risk prediction models beyond traditional markers.

Distinct ceramide molecular species were significantly associated with CVD among patients with coronary artery disease[13]. More recently, distinct serum ceramides were shown to be elevated in diabetes[28,29], and some specific ceramides may predict incident type 2 diabetes mellitus event years before the onset of the disease[17,30,31]. Data from these studies may help identify the clinical utility of ceramides for clinical testing. Measurement of serum ceramide can provide an independent and added value predictor to the routinely used diagnostic and prognostic CVD tools. However, the current results do not yet demonstrate causality. It is suggested that ceramides are associated with plaque susceptibility because they are known to promote many central atherosclerotic processes, including lipoprotein aggregation and absorption, inflammation, superoxide anion production, and apoptosis[32-34].

Plasma triglyceride concentrations primarily reflect the circulating burden of triglyceride-rich lipoproteins, including very-low-density lipoproteins (VLDL) and their remnants, which also carry substantial amounts of cholesterol. In the present study, triglycerides were analyzed as a surrogate of triglyceride-rich lipoprotein burden; remnant cholesterol was not directly measured.

An important limitation of the present study is its cross-sectional design, which precludes causal inference regarding the relationship between ceramides and CIMT. Moreover, residual confounding is possible because several established cardiovascular risk factors (such as smoking status, diabetes mellitus, and antihypertensive treatment) were not consistently available/complete for inclusion in the multivariable models. Future studies with more comprehensive risk-factor ascertainment should evaluate whether ceramides provide incremental value beyond fully adjusted conventional risk models. While associations were found , the results do not establish whether ceramide levels contribute to atherogenesis, are consequences of vascular disease, or reflect shared underlying metabolic pathways. In addition, CIMT is used mainly as a marker of subclinical atherosclerosis in epidemiological research and does not directly quantify atherosclerotic plaque burden. Although CIMT may support risk reclassification in some settings, its utility for individual patient risk stratification in routine clinical practice is limited; accordingly, the present findings should not be interpreted as supporting CIMT-based individualized clinical decision-making outside established multivariable risk assessment tools. Finally, the incomplete explained variance of CIMT highlights the multifactorial nature of vascular disease and the potential contribution of genetic, environmental, and psychosocial factors[35].

CONCLUSION

In this cross-sectional cohort, specific ceramide species were associated with CIMT, and a ceramide-based model showed explanatory and quartile-classification performance comparable to a conventional lipid-based model. Cer(d18:1/18:0) was inversely associated with CIMT, whereas Cer(d18:1/24:1) was positively associated. Because the study is cross-sectional, the findings should be interpreted as associations and do not support causal inference.These findings support further investigation of ceramides as complementary biomarkers related to subclinical vascular changes. Longitudinal studies in diverse populations are needed to clarify causal pathways, evaluate clinical utility, and determine whether ceramide profiling adds incremental value beyond established risk assessment approaches.

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Author contributions: JS: writing – original draft, data curation; GN: data analysis, visualization; IMRS: lipidomic analysis and final manuscript validation; JPGR: data curation; RP, MV, RB: final manuscript validation; JP: manuscript preparation/correction, validation, supervision.

Ethics approval: The study protocol adhered to the Helsinki declaration and all participants signed the informed consent. The Kardiovize Brno 2030 study was approved by the ethics committee of St Anne's University Hospital, Brno, Czech Republic (reference number 2 G/2012) (ref.[21]).

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request and with compliance to the General Data Protection Regulation.

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