Lipidomics Profiling and Risk of Cardiovascular Disease in the Prospective Population-Based Bruneck Study

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Background—The bulk of cardiovascular disease risk is not explained by traditional risk factors. Recent advances in mass spectrometry allow the identification and quantification of hundreds of lipid species. Molecular lipid profiling by mass spectrometry may improve cardiovascular risk prediction.

Methods and Results—Lipids were extracted from 685 plasma samples of the prospective population-based Bruneck Study (baseline evaluation in 2000). One hundred thirty-five lipid species from 8 different lipid classes were profiled by shotgun lipidomics with the use of a triple-quadrupole mass spectrometer. Levels of individual species of cholesterol esters (CEs), lysophosphatidylcholines, phosphatidylcholines, phosphatidylethanolamines (PEs), sphingomyelins, and triacylglycerols (TAGs) were associated with cardiovascular disease over a 10-year observation period (2000–2010, 90 incident events). Among the lipid species with the strongest predictive value were TAGs and CEs with a low carbon number and double-bond content, including TAG(54:2) and CE(16:1), as well as PE(36:5) (P=5.1×10^{-7}, 2.2×10^{-4}, and 2.5×10^{-3}, respectively). Consideration of these 3 lipid species on top of traditional risk factors resulted in improved risk discrimination and classification for cardiovascular disease (cross-validated ΔC index, 0.0210 [95% confidence interval, 0.0010-0.0422]; integrated discrimination improvement, 0.0212 [95% confidence interval, 0.0031-0.0406]; and continuous net reclassification index, 0.398 [95% confidence interval, 0.175-0.619]). A similar shift in the plasma fatty acid composition was associated with cardiovascular disease in the UK Twin Registry (n=1453, 45 cases).

Conclusions—This study applied mass spectrometry-based lipidomics profiling to population-based cohorts and identified molecular lipid signatures for cardiovascular disease. Molecular lipid species constitute promising new biomarkers that outperform the conventional biochemical measurements of lipid classes currently used in clinics. **(*Circulation*. 2014;129:1821-1831.)**

Key Words: biomarkers ■ cardiovascular diseases ■ lipids ■ mass spectrometry ■ metabolomics

There is a clear need for new biomarkers capable of identifying patients at risk of plaque destabilization and rupture.[1] Among various pathological features of atherosclerotic plaques that define their propensity to rupture, the content and composition of plaque lipids deserve special consideration.[2] Altered lipid metabolism and dyslipidemia in the context of inflammation and oxidative stress are driving forces in the transition from stable to unstable plaques. We have recently performed comparative lipidomics profiling of carotid endarterectomy specimens from symptomatic versus asymptomatic patients[3] and highlighted the existence of a characteristic lipid signature within unstable human plaques. This prompted us to further investigate molecular lipid profiles in the circulation.

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Mass spectrometry (MS) is the preferred method for in-depth studies of lipid-related pathomechanisms. Although nuclear magnetic resonance spectroscopy is fast and cheap,[4] it offers limited possibilities to resolve individual lipid species.[5] In contrast, shotgun lipidomics with MS can screen and simultaneously analyze molecular lipid species in nonseparated lipid extracts.[6] Recent advances in MS allow the application of this technology to epidemiological cohorts of cardiovascular disease (CVD).[7,8]

In this study, we performed lipidomics profiling in the prospective population-based Bruneck Study and analyzed the

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association of 135 distinct lipid species with CVD risk over a 10-year observation period. The profiles of circulating lipid species were then compared with those previously described in atherosclerotic plaques.[3] External validation for the observed shift in fatty acid (FA) composition was performed in the UK Twin Registry.

Methods

Study Subjects and Examination

The Bruneck Study is a prospective, population-based survey of the epidemiology and pathogenesis of atherosclerosis and CVD.[9] At the 1990 baseline evaluation, the study population comprised an age- and sex-stratified random sample of all inhabitants of Bruneck (125 men and 125 women from each of the fifth through eighth decades of age, all white). In 2000, 702 subjects were still alive and participated in the second quinquennial follow-up. As part of the 2000 follow-up, citrate plasma samples were drawn after an overnight fast and 12 hours of abstinence from smoking. Samples were divided into aliquots and immediately stored at −80°C. Plasma samples for lipidomics analyses were available for 685 individuals (97.6%). During the follow-up between 2000 and 2010, detailed information about fatal and nonfatal new-onset CVD was carefully collected (follow-up, 100% complete). The study protocol was approved by the ethics committees of Bolzano and Verona and conformed to the Declaration of Helsinki, and all study subjects gave their written informed consent. Risk factors were assessed as outlined in the expanded Methods in the online-only Data Supplement. The composite CVD end point included incident fatal and nonfatal myocardial infarction, ischemic stroke, and sudden cardiac death. The presence of myocardial infarction was assessed by World Health Organization criteria,[10] and ischemic stroke was classified according to the criteria of the National Survey of Stroke.[11]

MS Analysis

MS analysis was performed as previously described.[3] Lipid species were measured in the entire Bruneck cohort based on full MS and 5 neutral loss (NL 141.0, NL 185.0, NL 213.0) and precursor ion (PI 184.1, PI 369.3) scans that contained the abundant lipid species. Sample processing and data processing are detailed in the expanded Methods in the online-only Data Supplement.[12,13]

Statistical Analysis

Cox proportional hazards models were built to assess the association of each individual lipid species with CVD risk. The proportional hazards assumption was tested by computing the significance level of the correlation coefficient between Kaplan-Meier-transformed survival time and scaled Schoenfeld residuals for all variables in all models. Adjustment for multiple testing was performed by means of the Benjamini-Hochberg procedure, which is more appropriate in an "–omics" setting than the Bonferroni correction.[14] Results were very similar after the concentrations of lipid species were log-transformed. For ease of interpretation, only results derived from untransformed data are presented. Potential undue influence of outliers was examined by rerunning analyses after the exclusion of cases with lipid concentrations >3 SDs higher or lower than the mean. This approach again produced almost identical results. For comparison purposes, we calculated the sum of all individual lipid species within each lipid class and the respective first principal components.

For variable selection in the setting of high data dimensionality and extensive intercorrelations, L_1-regularized Cox regression was used, which implements the least absolute shrinkage and selection operator (LASSO) algorithm (see the expanded Methods in the online-only Data Supplement).[15] This model considered all lipid species simultaneously, and age, sex, and statin use were included as unpenalized explanatory variables. The optimal hyperparameter λ was chosen as the one maximizing the partial likelihood as determined by 10-fold cross-validation. To account for the potentially large variance of cross-validation, this process was repeated 1000 times with different data partitions. Two analyses with alternative selection procedures were run for validation purposes (see the expanded Methods in the online-only Data Supplement). Multivariable effect sizes and confidence intervals (CIs) for the final LASSO selection of lipid species were calculated by conventional unpenalized Cox regression to facilitate traditional formal inference.

The incremental predictive value of selected lipid species for CVD risk prediction was assessed by measures of risk discrimination (changes in the Harrell C index)[16] and risk reclassification (categorical),[17] continuous and prospective net reclassification indexes[18] (NRIs), integrated discrimination improvement,[17] and relative integrated discrimination improvement.[17] Estimates of incremental predictive value and their CIs were cross-validated (3000 repetitions) and are therefore estimates of expected out-of-sample predictive performance.

The calculation of categorical NRIs was based on 10-year risk categories of <0.050, 0.050 to 0.075, and >0.075 according to the most recent American College of Cardiology/American Heart Association blood cholesterol guidelines.[19] This analysis considered 90 participants who experienced a CVD event and 498 who remained free of CVD throughout the entire 10-year survey period.[17] The prospective variant of the categorical NRI[18] additionally included participants who were censored before year 10 because of causes unrelated to CVD (n=97). The continuous NRI[17] does not depend on the choice of categories but deems any change in predicted risk in the correct direction as appropriate. We evaluated the improvement in CVD risk prediction when selected lipid species were used (1) in addition to conventional risk factors (age, sex, diabetes mellitus, smoking status, systolic blood pressure, total cholesterol, and high-density lipoprotein cholesterol) and (2) in replacement of standard lipid components in the Framingham Risk Score. Further details are given in the expanded Methods in the online-only Data Supplement. Analyses were performed with R 2.15.1 and STATA 12; graphics were created with the ggplot2 package.[20]

Results

Molecular Lipid Profiling in the Bruneck Study

Baseline demographic, clinical, and laboratory characteristics of the 685 participants (Bruneck Study) are shown in Table I in the online-only Data Supplement. Over a follow-up period of 10 years (5902 person-years), 90 participants experienced a CVD event, corresponding to an incidence rate of 15.2 (95% CI, 12.4–18.7) per 1000 person-years. Shotgun lipidomics detected 135 lipid species attributable to 8 different lipid classes: phosphatidylcholine (PC), lysophosphatidylcholine (LPC), cholesterol ester (CE), sphingomyelin (SM), phosphatidylserine (PS), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), and triacylglycerol (TAG). Details are provided in Table II in the online-only Data Supplement. Plasma levels of the individual lipid species were strongly correlated with each other (Figure I in the online-only Data Supplement). Figure 1 shows significance levels for the associations of individual lipid species (dots) and 2 lipid class summary measures (bars) with incident CVD (logarithmic *y* axis). A number of individual lipid species within the classes of TAG, CE, LPC, and PC outperformed the respective summary measures.

Associations of Lipid Species With CVD Risk

Considering each lipid species separately, 50 members of TAGs, CEs, PEs, PCs, LPCs, and SMs were significantly associated with CVD risk (Table III in the online-only Data Supplement). When controlling for multiple comparisons, 28 lipids maintained significance (Table III in the online-only Data Supplement). On comparing these lipid species with lipids characteristic of advanced atherosclerotic plaques,[3] we found

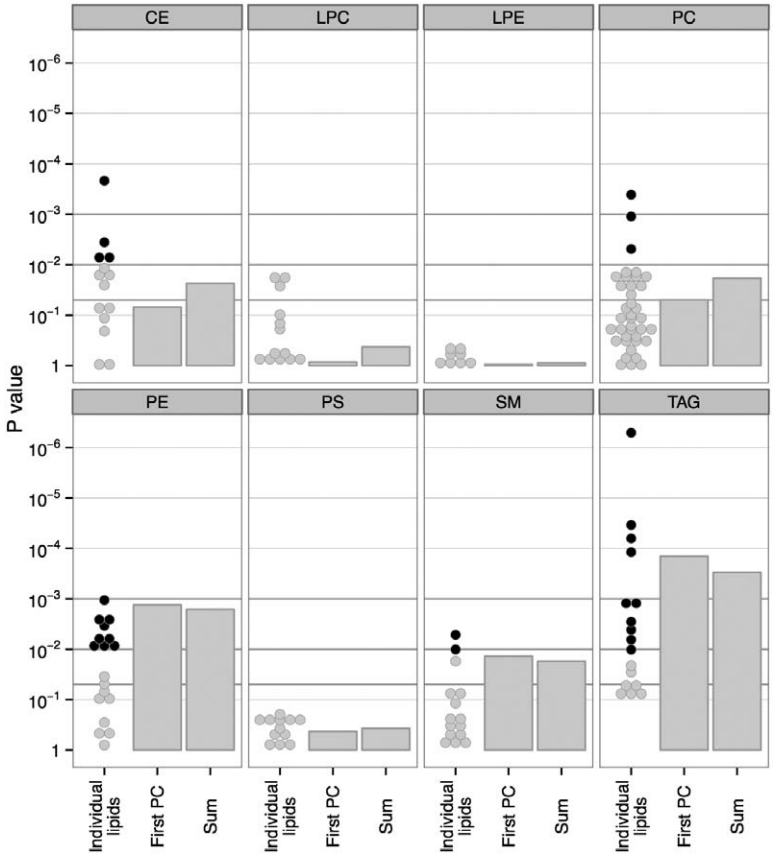

Figure 1. Manhattan plot depicting significance levels (logarithmic *y* axis) for the association of individual lipid species and lipid groups with incident cardiovascular disease (CVD). The composite CVD end point considers myocardial infarction, ischemic stroke, and sudden cardiac death (Bruneck Study, 2000–2010, 90 events). Individual lipids are visualized as dots; lipid groups, as bars. Lipid classes are shown in separate panels. *P* values were derived from Cox proportional hazards models including the respective lipid species, age, sex, and statin therapy. Lipids maintaining significance after adjustment for multiple testing by the Benjamini-Hochberg procedure, controlling the false-discovery rate at the 0.05 level, are marked with black dots. CE indicates cholesteryl ester; First PC, first principal component; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; Sum, the sum of all lipids within a group; and TAG, triacylglycerol.

a broad overlap (enrichment analysis, *P*=0.047). For example, PC(38:3), the only PC that was not detectable in control arteries but was identified in atherosclerotic lesions showed a strong association with CVD in the Bruneck Study. Additionally, the group of relevant plasma CE species included a proportion of plaque-enriched CEs as high as 50%, which surpassed the proportions observed for other lipid classes (*P*=0.04). These findings support the concept that circulating lipids reflect the disease process in the atherosclerotic tissue.

In Figure 2, all 135 lipid species are plotted as circles with their position in the 2-dimensional lipid class graphs determined by the total acyl chain carbon numbers (*x* axis) and double-bond content (*y* axis). The lipid species highlighted with a yellow halo were previously identified in atherosclerotic plaques but not in control arteries and thus may be viewed as plaque-enriched lipid species (see above).[3] The color of each circle depicts the strength and direction (positive or negative) of the given associations. The size of the circle indicates the level of statistical significance. Of note, associations were most pronounced for TAGs and CEs of lower carbon number and double-bond content (ie, saturated and monounsaturated FAs), and the risk profile was complemented by PE/PCs, SMs (both positive) and LPCs (negative). Full details are given in Table III in the online-only Data Supplement. Subgroup analyses were performed in men and women (Figure II in the online-only Data Supplement) and age strata. These analyses did not yield evidence of differential effects (the number of interaction terms with a value of *P*<0.05 was lower than that expected by chance: 2 and 3 versus 7) and justify pooled analysis of sexes and of the entire age range. As expected, statin therapy had some effect on the association patterns (Figure III in the online-only Data Supplement). Thus, analyses were adjusted for statin use, and key computations were repeated after the exclusion of participants on statins.

Lipidomics Signature for CVD Risk

To identify a lipidomics signature of CVD risk, we applied LASSO and 2 alternative selection algorithms and network inference as backup methods. In L_1-regularized Cox regression analysis, the 3 lipid species TAG(54:2), CE(16:1), and PE(36:5) were most consistently related to incident CVD (Figure IV in the online-only Data Supplement). Details on inclusion fractions in 1000 runs are provided in Table 1 and compared with models using backward selection and best subset algorithms. The last 2 approaches selected more lipid species among TAG, CE, PE, PC, and LPC classes, but the key selection of LASSO was well represented (Table 1 and Table IV in the online-only Data Supplement). Multivariable effect sizes and CIs for the LASSO selection of lipid species are shown in Figure 3. After the exclusion of 65 subjects taking statins, LASSO still selected TAG(54:2) and CE(16:1) but gave preference to PC(32:1) over PE(36:5) within the PE/PC cluster.

Lipid Network Analysis

Differences in the 3 lipid species TAG(54:2), CE(16:1), and PE(36:5) were reflected in the modules of the network inference analysis (Figure 4). As expected, the module containing all TAG species (Figure 4, blue module) was strongly correlated

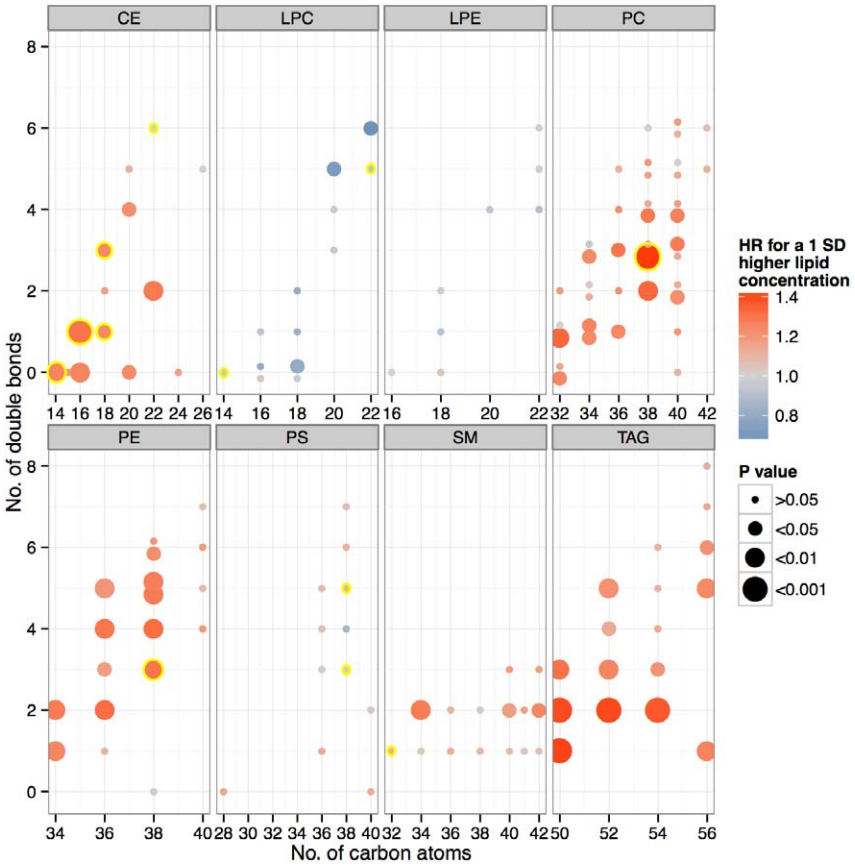

Figure 2. Associations of 135 lipid species with incident cardiovascular disease (CVD). The composite CVD end point considers myocardial infarction, ischemic stroke, and sudden cardiac death (Bruneck Study, 2000–2010, n=90 events). Individual lipid species are depicted by filled circles and arranged by lipid class in 8 panels according to the number of total carbon atoms (*x* axes) and number of double bonds (*y* axes). Lipid species highlighted with a yellow halo were detectable in atherosclerotic plaques but not in healthy control arteries (plaque-enriched lipid species).[3] No yellow halos are given for triacylglycerol for reasons detailed in the online-only Data Supplement. Circle color indicates the magnitude of hazard ratio (HR), and circle size corresponds to the significance level (see the legend). HRs were calculated for a 1-SD unit higher lipid concentration and derived from Cox proportional hazards models with adjustment for age, sex, and statin therapy. Lipids with the same number of carbon atoms and double bonds are pulled apart vertically to increase their visibility. The distinguishing feature in this case is the presence of an alkyl ether linkage, signified in the formula as, eg, PC(O-38:3). Lipids possessing such a linkage are pulled upward, and their alkyl-ether-free counterparts are pulled downward. CE indicates cholesteryl ester; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; and TAG, triacylglycerol.

with total triglyceride levels (Pearson correlation coefficient, 0.86; Figure V in the online-only Data Supplement). Similarly, total and low-density lipoprotein cholesterol showed strong correlations with the modules containing the bulk of CE and PC/PE species (Pearson correlation coefficients, 0.38 – 0.55; Figure 4, black, green, turquoise, and yellow modules). The connectivity within and between modules was calculated for each lipid, and the TAGs showed a high intraconnectivity and a low interconnectivity compared with the other lipids and modules (Table V in the online-only Data Supplement). TAG(54:2) had one of the largest differences (1.83) between the intraconnectivity and interconnectivity between the modules, whereas CE(16:1) had one of the lowest differences (−0.25) and PE(36:5) was in between (0.03). The proportions of connections to lipid species associated with CVD were 30% for CE(16:1), 61% for TAG(54:2), and 83% for PE(36:5).

CVD Risk Prediction and Classification

Only cross-validated estimates of incremental predictive value are presented throughout this study. The addition of TAG(54:2), CE(16:1), and PE(36:5) to a model including conventional risk factors increased the C index by 0.0210 (95% CI, 0.0010–0.0422) and yielded an integrated discrimination improvement of 0.0212 (95% CI, 0.0031–0.0406). Corresponding data for the addition of 6 lipid species were 0.0376 (95% CI, 0.0065–0.0697) and 0.0337 (95% CI, 0.0072–0.0615). NRIs were calculated for the 10-year risk categories of <0.050, 0.050 to 0.075, and >0.075 and are summarized in Table 2. In brief, consideration of TAG(54:2), CE(16:1), and PE(36:5) on top of conventional risk factors resulted in a significant improvement in risk stratification (NRI=0.087; 95% CI, 0.016–0.159) that was driven mainly by correct reclassification of noncases (NRI=0.068; 95% CI, 0.030–0.106). Similarly, replacement of standard lipid measures of the Framingham Risk Score (total and high-density lipoprotein cholesterol) with TAG(54:2), CE(16:1), and PE(36:5) significantly improved risk discrimination and 10-year risk reclassification (Table 2). Findings were similar after exclusion of subjects with prior CVD (Table VI in the online-only Data Supplement).

Table 1. Inclusion Fractions of the 10 Individual Lipid Species Most Frequently Selected in 3 Different Selection Procedures

LASSO		Best Subset		Stepwise	
Variable	Selected, %	Variable	Selected, %	Variable	Selected, %
CE(16:1)*	100.0*	TAG(54:2)*	99.7	PE(36:5)*	72.4
TAG(54:2)*	100.0*	CE(16:1)*	43.7	TAG(54:2)*	69.5
PE(36:5)*	95.3*	PC(O-34:1)	35.1	TAG(50:3)	68.2
SM(34:2)	51.9	LPC(18:1)	34.7	TAG(52:5)	66.3
LPC(20:5)	25.3	PE(36:5)*	28.3	TAG(52:2)	62.6
LPC(22:6)	19.6	TAG(54:3)	26.1	CE(16:1)*	56.0
PE(38:3)	4.2	PS(38:4)	23.5	PE(O-38:5)	53.6
LPC(18:1)	1.3	PC(38:6)	22.6	TAG(50:2)	48.5
PC(32:1)	1.3	TAG(52:2)	15.4	PC(32:1)	47.0
PC(O-34:1)	1.3	PC(32:0)	11.5	TAG(54:3)	46.1

Age, sex, and statin medication were included in all models. LASSO: All 135 lipids were standardized to have unit variance. One thousand repetitions of L_1-regularized Cox regression with the optimal degree of regularization determined by partial likelihood cross-validation were performed. Inclusion fractions refer to the fraction of models in which the respective lipid had a nonzero coefficient. Best Subset: Prescreening was performed on all 135 lipids by backward stepwise Cox regression and reduced the number of candidate lipids to 46. Cox models were then fitted to all combinations of up to 6 of these 46. Of the resulting 10.9 million models, the 1000 with the lowest Bayesian information criterion were selected. Inclusion fractions refer to the fraction of models containing the respective lipid. Stepwise: For each of 1000 bootstrap resamples, (1) all 135 lipids were prescreened with Cox regression, eliminating lipids with a $P<0.05$, and (2) backward stepwise Cox regression with minimum Akaike information criterion as selection criterion was performed on the remaining lipids. Inclusion fractions refer to the fraction of the 1000 final models containing the respective lipid. A detailed description of the selection strategies is provided in the expanded Methods section in the online-only Data Supplement. For each selection approach, the 10 most frequently selected lipids are shown. CE indicates cholesteryl ester; LASSO, least absolute shrinkage and selection operator; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; and TAG, triacylglycerol.

*The key selection of LASSO was well represented within these choices of the other 2 approaches. Full data are given in Table IV in the online-only Data Supplement.

Comparison With the TwinsUK Cohort

Samples from the TwinsUK Cohort (n=1453, 45 CVD cases) were used for validation purposes. In the Bruneck Study, there was a clear shift in the chain length of FA among lipid species associated with CVD risk, especially for CE, SM and TAG. These 3 classes accounted for the majority of plasma lipids and thus dominated the FA content in complex lipids (Figure 5A). On the basis of the different combinations of FAs, which could combine to create the TAGs with statistical significance in the Bruneck cohort (TAG[50:1], TAG[50:2], TAG[50:3], TAG[52:2], TAG[52:3], TAG[52:5], TAG[54:2], TAG[56:1], TAG[56:5], and TAG[56:6]), there was a high frequency of myristate (14:0), palmitate (16:0), stearate (18:0), myristoleate (14:1), palmitoleate (16:1), and oleate (18:1). Similarly, in CEs and SMs related to CVD risk in the Bruneck Study, the most abundant FAs were myristate (14:0), palmitate (16:0), palmitoleate (16:1), and oleate (18:1; Figure 5A). For replication, we determined the free FA composition in plasma samples of the

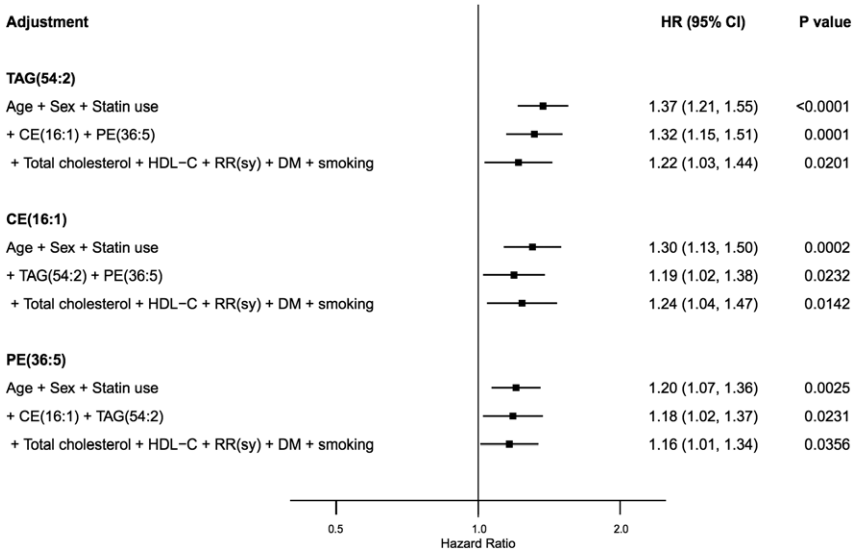

Figure 3. Multivariable effect estimates and confidence intervals (CIs) for the 3 lipid species most consistently associated with incident cardiovascular disease (CVD). The composite CVD end point considers myocardial infarction, stroke, and sudden cardiac death (Bruneck Study, n=90). Hazard ratios (HRs) and 95% CIs were derived from standard Cox regression models with progressive adjustment and calculated for a 1-SD-higher lipid level. CE indicates cholesteryl ester; DM, diabetes mellitus; HDL-C, high-density lipoprotein cholesterol; HR, hazard ratio; PE, phosphatidylethanolamine; RR(sy), systolic blood pressure; and TAG, triacylglycerol.

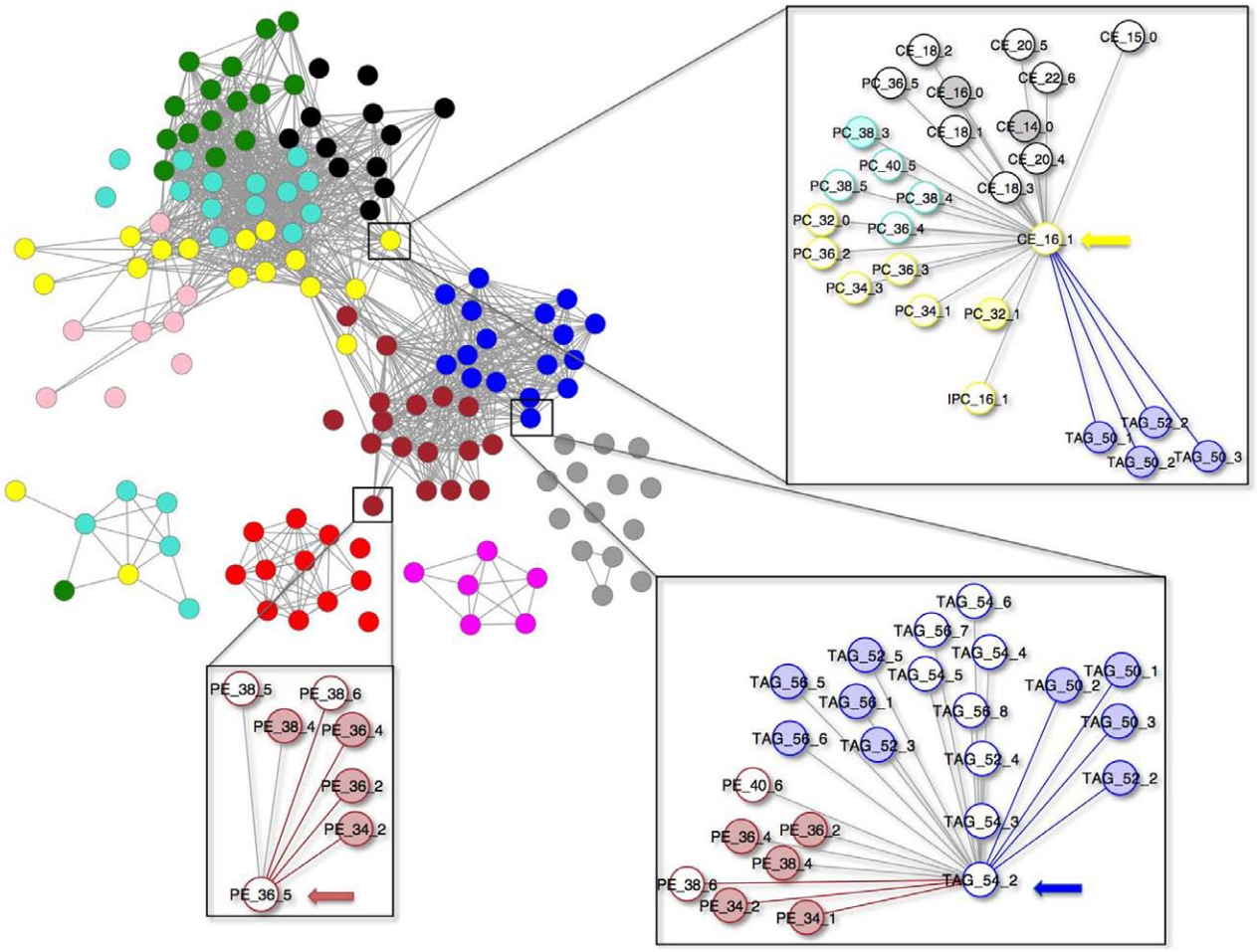

Figure 4. Lipid network analysis. The lipid correlation network, thresholded at an adjacency of 0.02 (akin weighted correlation of 0.8), is shown. The colors indicate the modules detected by topological overlap measure. Although many of the lipid species within the same class belong to the same module, there are modules that contain lipids from >1 lipid class. The direct neighbors of the 3 lipid species CE(16:1), TAG(54:2), and PE(36:5) are shown in the insets. Lipid species associated with CVD in the Bruneck Study are depicted as colored nodes. Lipid molecules directly connected to either CE(16:1) and TAG(54:2) or PE(36:5) and TAG(54:2) are highlighted by colored edges. For details about the connectivity of lipid species, refer to Table V in the online-only Data Supplement. CE indicates cholesteryl ester; PE, phosphatidylethanolamine; and TAG, triacylglycerol.

TwinsUK Cohort by MS (Figure 5B). In close agreement with the data from the Bruneck study, levels of myristate (14:0), palmitate (16:0), palmitoleate (16:1), and oleate (18:1) were more strongly associated with CVD than other FAs.

Discussion

This is the first population-based study reporting a systematic analysis of the plasma lipidome in the context of CVD. It allows 4 main conclusions: (1) There is a broad diversity of potential cardiovascular effects of lipid species within most lipid classes, and as a consequence, individual lipids outperform lipid summary measures with regard to CVD risk prediction (Figure 1); (2) TAGs of low carbon number and double-bond content show the strongest and most consistent associations with CVD, surpassing CEs and PE/PCs (Figure 2); (3) molecular lipid profiling by MS results in a significant improvement in CVD risk discrimination and classification beyond the information provided by classic risk factors, including conventional lipid measures (Table 2); and (4) the stronger association of certain lipid species with CVD can, at least in part, be explained by a shift in the plasma FA composition (Figure 5).

Postgenomics Technologies

Recent advances in postgenomics technologies allow the interrogation of CVD at the transcriptome (RNA), proteome (proteins), and metabolome (small molecules) levels. Previous metabolomic studies have suggested significant associations between CVD and branched chain amino acids, acetylcarnitines, free FAs,[21,22] and metabolites linked to choline metabolism.[23] We assessed 135 lipid species in a prospective population-based cohort using shotgun lipidomics. Lipids are among the main culprits in vessel pathology and thus represent a prime target for metabolomic profiling in cardiovascular research. Indeed, several lipid species within 6 of 8 lipid classes-TAG, CE, PE, PC, SM, and LPC-showed significant associations with future CVD. Importantly, lipidomics interrogated information that was not captured by the established CVD risk factors based on the conventional biochemical measurements of triglycerides, total cholesterol, and high- and low-density lipoprotein cholesterol only.

Triglyceride Species and CVD

A specific cluster of TAGs with low carbon number and double-bond content, that is, saturated and monounsaturated

Table 2. Improvement in 10-Year Risk Prediction and Risk Classification by Lipid Species Derived From Plasma Lipidomics

Model	Cross-Validated Performance Statistic (95% Confidence Interval)		
	C index	C Index Change	Continuous NRI*
Conventional risk factors†	0.714 (0.662 to 0.764)	Reference	Reference
+TAG(54:2), PE(36:5), CE(16:1)	0.735 (0.687 to 0.781)	0.0210 (0.0010 to 0.0422)	0.398 (0.175 to 0.619)
Above+SM(34:2), LPC(20:5), LPC(22:6)	0.751 (0.707 to 0.794)	0.0376 (0.0065 to 0.0697)	0.406 (0.183 to 0.625)
Conventional risk factors†		Reference	Reference
+3 lipids replacing HDL-C and T-C	0.739 (0.691 to 0.786)	0.0253 (0.0026 to 0.0492)	0.303 (0.080 to 0.525)
+6 lipids replacing HDL-C and T-C	0.754 (0.709 to 0.797)	0.0399 (0.0076 to 0.0731)	0.363 (0.140 to 0.584)
	Categorical NRI*‡	Categorical NRI Noncases*‡	Categorical NRI cases*‡
Conventional risk factors†	Reference	Reference	Reference
+TAG(54:2), PE(36:5), CE(16:1)	0.087 (0.016 to 0.159)	0.068 (0.030 to 0.106)	0.019 (–0.040 to 0.080)
Above+SM(34:2), LPC(20:5), LPC(22:6)	0.149 (0.065 to 0.234)	0.127 (0.081 to 0.174)	0.022 (–0.048 to 0.093)
Conventional risk factors†	Reference	Reference	Reference
+3 lipids replacing HDL-C and T-C	0.081 (0.004 to 0.160)	0.053 (0.014 to 0.093)	0.028 (–0.039 to 0.096)
+6 lipids replacing HDL-C and T-C	0.147 (0.061 to 0.234)	0.109 (0.061 to 0.157)	0.038 (–0.034 to 0.112)
	Prospective NRI‡	IDI	Relative IDI
Conventional risk factors†	Reference	Reference	Reference
+TAG(54:2), PE(36:5), CE(16:1)	0.080 (0.013 to 0.149)	0.0212 (0.0031 to 0.0406)	0.217 (0.033 to 0.442)
Above+SM(34:2), LPC(20:5), LPC(22:6)	0.141 (0.060 to 0.222)	0.0337 (0.0072 to 0.0615)	0.346 (0.071 to 0.740)
Conventional risk factors†	Reference	Reference	Reference
+3 lipids replacing HDL-C and T-C	0.075 (0.000 to 0.151)	0.0234 (0.0034 to 0.0448)	0.241 (0.036 to 0.505)
+6 lipids replacing HDL-C and T-C	0.139 (0.057 to 0.224)	0.0329 (0.0059 to 0.0609)	0.338 (0.058 to 0.736)

The sample comprised 685 participants and 90 cardiovascular disease events. In addition, 3 lipids indicates TAG(54:2), PE(36:5), and CE(16:1); and 6 lipids, TAG(54:2), PE(36:5), CE(16:1), SM(34:2), LPC(20:5), and LPC(22:6). Estimates are means of 3000 repetitions of 5-fold cross-validation. Confidence intervals are percentile bootstrap intervals based on 1000 repetitions and were cross-validated in the same way. C index indicates concordance index; HDL-C, high-density lipoprotein cholesterol; IDI, integrated discrimination improvement; LPC, lysophosphatidylcholine; NRI, net reclassification improvement; PE, phosphatidylethanolamine; SM, sphingomyelin; TAG, triacylglycerol; and T-C, total cholesterol.

*Calculation of retrospective NRI was based on 90 cases and 498 noncases.

†Models considering the standard Framingham Risk Score components age, sex, history of diabetes mellitus, smoking status, systolic blood pressure, total cholesterol, and HDL cholesterol.

‡Categorical retrospective and prospective NRI were calculated according to risk cutoffs of 0.050 and 0.075.

acyl chains, was most consistently associated with CVD (lowest $P=4.6\times10^{-7}$; Figures 1 and 2). This observation corroborates a recent lipidomics survey that identified a similar set of TAGs rather than total triglycerides as risk predictors of type 2 diabetes mellitus,[24] suggesting that the relevance of TAGs in the context of both diseases may have been underestimated in previous research by an unwarranted focus on total triglycerides. Large meta-analyses have yielded solid evidence of an association between triglycerides and CVD,[25] but it remains controversial whether triglyceride levels are just a marker of proatherogenic lipoprotein dynamics and composition[26,27] or are causally related to lipoprotein retention in the vessel wall, plaque stability, and thrombogenicity.[27,28] Our finding that certain TAG species rather than total triglycerides confer increased CVD risk and intriguing data from recent Mendelian randomization analyses[29] support the latter view.

CE Species and CVD

As expected, CEs showed significant associations with incident CVD. Similar to TAGs, the most strongly associated CEs had a low carbon number and double-bond content (Figure 2). Notably, the top-ranked CE in our study, cholesteryl palmitoleate CE(16:1), was among the few CE species that were detectable only in advanced atherosclerotic plaques but not in normal arteries.[3] CE(16:1) is derived from acyl-CoA:cholesterol acyl transferase-2 activity and showed significant associations with incident myocardial infarction and acute coronary syndrome in 2 previous analyses.[30,31] Interestingly, lipid profiling in transgenic mice identified palmitoleate (16:1) as an adipose tissue-derived lipid hormone that can modulate systemic insulin sensitivity.[32] It is also one of the main products in the endogenous synthesis of nonessential FAs,[33] whereby acetyl-coenzyme A is polymerized to form myristate (14:0), elongated to the initial major product palmitate (16:0), and desaturated to palmitoleate (16:1). Both, CE(14:0) and CE(16:0) showed significant, albeit weaker, associations with incident CVD in the Bruneck study (Table III in the online-only Data Supplement).

Other Lipid Species and CVD

Besides CEs and TAGs, several PE/PC species and few SMs were linked to CVD risk. For example, the only PC that was exclusively identified in atherosclerotic plaques,[3] PC(38.3), showed a strong association with CVD in the Bruneck Study (Figure 2). Similarly, SM(34:2) was positively associated with CVD, which is consistent with the shift in the FA

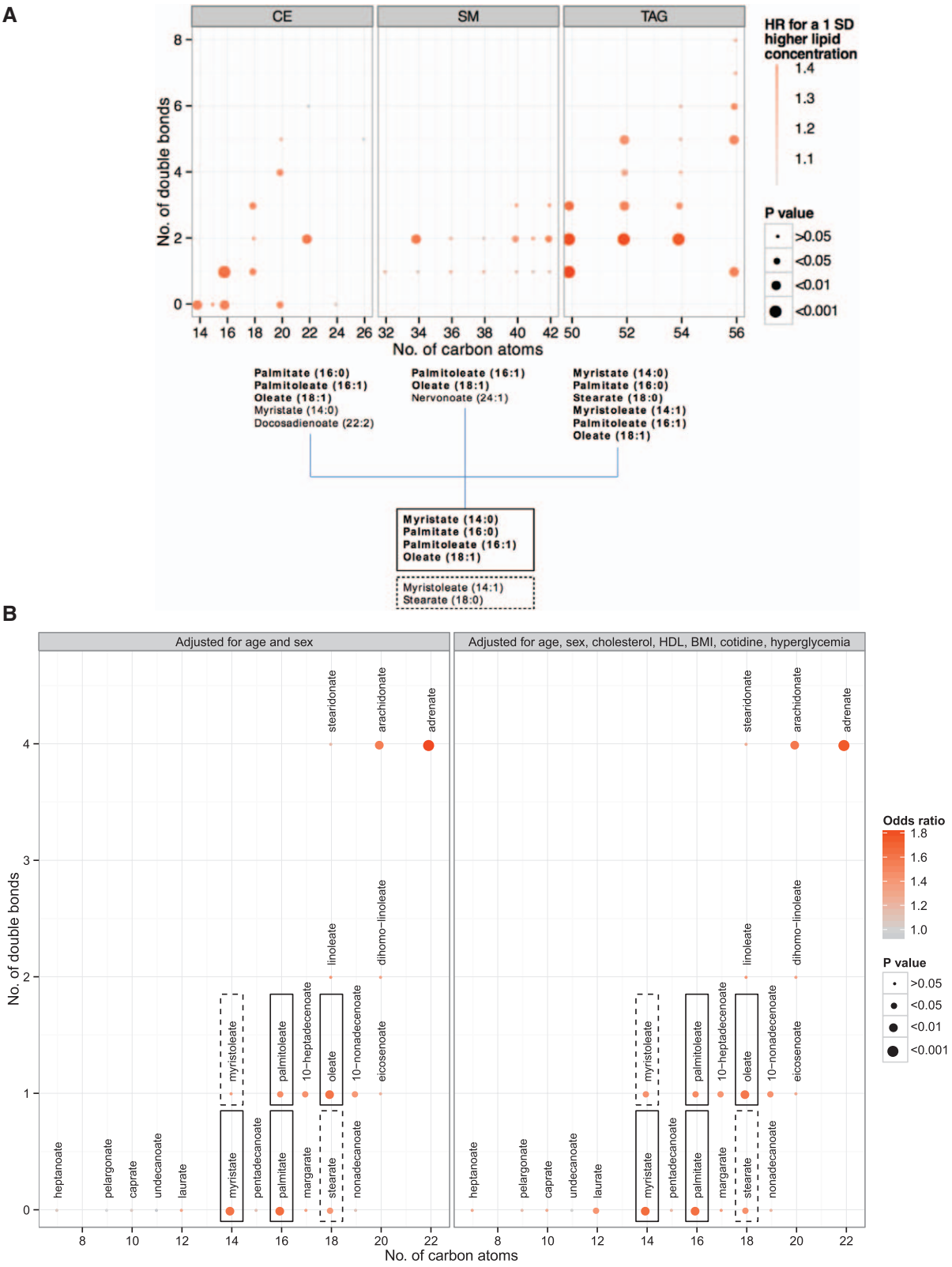

Figure 5. Replication in the TwinsUK Cohort. **A**, Fatty acid (FA) composition of lipid species associated with cardiovascular disease (CVD) in the Bruneck Study from the cholesteryl ester (CE), sphingomyelin (SM), and triacylglycerol (TAG) classes, which accounted for >80% of plasma lipids and thus dominated the FA pool in complex lipids. This figure highlights the most abundant FAs in the respective groups (bold) considering the quantity of the individual lipid species as detailed in Table III in the online-only Data Supplement. FA composition in TAGs was estimated by a simulation analysis considering all combinations of long-chain FAs that resulted in the correct carbon and double-bond number. FAs highlighted in the box were most consistently found in lipid species associated with CVD in the Bruneck study. **B**, Associations of plasma FA with CVD in the TwinsUK Cohort (n=1453, 45 cases) with adjustment for age and sex only (**left**) or multivariate adjustment for age, sex, total cholesterol, high-density lipoprotein cholesterol, body mass index, current smoking (ascertained by cotidine levels), and diabetes mellitus (**right**). The key selection of FAs in the Bruneck Study is highlighted. HR indicates hazard ratio.

composition observed in other lipid classes, that is, palmitoleate (16:1) and oleate (18:1) in TAGs and CEs. These data may point to hitherto not sufficiently recognized pathophysiological lipid pathways in CVD; that is, de novo lipogenesis (14:0, 16:0, 18:0, 16:1, 18:1) results in an FA pattern very similar to that linked to CVD in complex lipids and appears to be associated with a higher CVD risk than conjugation with many essential FAs. In contrast, LPCs showed an inverse relationship. The latter finding is counterintuitive because proatherogenic lipoprotein-associated phospholipase A2 activity results in the generation of LPCs and LPC content is elevated in plaques.[3] However, it is in agreement with the findings of a recent study on coronary artery disease (CAD) and may reflect increased LPC catabolism and clearance from the circulation.[34] Moreover, circulating LPC is partially generated by lecithin-cholesterol acyltransferase, and low lecithin-cholesterol acyltransferase activity has been linked to CAD.[35,36]

Molecular Lipid Signature and CVD

Within the system-wide lipid network, 3 lipid species were most informative for CVD risk in our study: TAG(54:2), CE(16:1), and PE(36:5) (Figure 4 and Table III in the online-only Data Supplement). The addition of these lipid species to models containing standard CVD risk factors improved risk discrimination and stratification; the same was true when standard lipid measures were replaced by these 3 variables (Table 2). Strengths of our study include its size, prospective design, thorough characterization of study subjects, complete and high-quality assessment of outcome events, and consistency of findings in sensitivity analyses. Internal cross-validation (3000 repetitions) revealed significant improvement in expected out-of-sample risk prediction performance conferred by the top 3 lipids. Additional validation was performed by comparisons against the lipid composition of atherosclerotic plaques. External validation of the shift in FA composition was performed in the UK Twin Registry.

Only 1 epidemiological study on lipidomics and CVD has been published to date; Meikle et al[37] compared lipid profiles of patients with stable and unstable CAD and control subjects (n=220 overall) and revealed a plethora of differences. As in our study, most positive associations with both stable and unstable CAD were found for TAGs, followed by PE/PC, LPC, and CE species. Findings for CE differ. We obtained a positive association with CVD; in contrast, Meikle et al[37] demonstrated positive associations with stable CAD but inverse associations with unstable CAD. This may be explained by the fact that 88% of patients with unstable CAD but only 54% of patients with stable CAD had received statins.[37] Similarly, 32% of unstable but none of the stable CAD patients had received heparin,[37] a well-known activator of lipoprotein lipase.

Limitations and Future Directions

There is no single method that can resolve the entire complexity of plasma lipids. Shotgun lipidomics offers an overview of abundant lipid species[38,39] but not full coverage of the plasma lipidome.[40] In addition, isobaric lipid species cannot be resolved. Another drawback of the technique is measurement variability (Table II in the online-only Data Supplement), reflected in higher coefficients of variation, particularly for low-abundant lipid species like LPEs. This, however, would be expected to weaken evident associations rather than to create spurious ones (regression dilution bias). As a result of the use of a single internal standard per lipid class, the concentrations determined in shotgun lipidomics are approximate because the ionization efficiency and the fragmentation chemistry of individual lipid species within the class may differ. In particular, for neutral lipids, the ionization efficiency shows a dependence on alkyl chain length and degree of unsaturation. On the other hand, shotgun lipidomics allowed the simultaneous analyses of 135 lipid species in nonseparated lipid extracts using just 5 precursor ion/neutral loss scans in positive ion mode.[39,41] A key limitation of our study is the lack of replication cohorts with suitable lipidomics measurements for external validation of the predictive utility of the top selection of lipid species. Future studies must address this issue, refine the final selection of lipid species, establish easy-to-use and reproducible MS assays, and reassess the utility of these tests in terms of risk discrimination and classification.

Conclusions

Our findings challenge the current practice of lipid management with its main focus on cholesterol and lipid levels (best addressed by drug therapy) rather than lipid composition (potentially targetable by dietary measures).[42] They also highlight the urgent need for mechanistic studies to explore the distinct biochemical characteristics and cellular effects of lipid species instead of lipid classes and to scrutinize their potential role in plaque destabilization and manifestation of CVD. A better knowledge of the functional roles of various lipid molecules presents an opportunity to advance our understanding of the role of lipids in the origin of atherosclerosis and to establish novel lipid biomarkers and therapeutic targets beyond the traditional lipid measurements.

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Disclosures

King's College London filed patent applications on lipid species as cardiovascular biomarkers. The authors report no other conflicts.

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CLINICAL PERSPECTIVE

The bulk of cardiovascular disease risk is not explained by traditional risk factors. Mass spectrometry allows a detailed analysis of the molecular lipid composition in plasma. We applied a shotgun lipidomics method using a triple-quadrupole mass spectrometer to plasma samples of the prospective population-based Bruneck Study (baseline evaluation in 2000). In this shotgun lipidomics analysis, we detected 135 lipid species attributable to 8 different lipid classes. Levels of individual species of cholesterol esters, phosphatidylethanolamines, and triacylglycerols were associated with cardiovascular disease over a 10-year observation period (2000–2010, 90 incident events). Molecular lipid signatures, consisting of individual lipid species from different lipid classes, as measured by mass spectrometry resulted in a significant improvement in cardiovascular risk discrimination and classification beyond the information provided by classic risk factors. To the best of our knowledge, this is the first study to use advanced mass spectrometry-based lipidomics profiling in the primary preventive setting. The importance of individual lipid species in the context of cardiovascular disease may have been underestimated by an unwarranted focus on lipid classes, including the traditional lipid measures of total triglycerides, total cholesterol, and low- and high-density lipoprotein cholesterol.