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Increased Levels of Circulating Fatty Acids Are Associated with Protective Effects against Future Cardiovascular Events in Nondiabetics

Muhammad Anas Kamleh,[†,□,■] Olga McLeod,[‡,□,■] Antonio Checa,[†,□,■] Damiano Baldassarre,[§,∥,■] Fabrizio Veglia,[∥,■] Karl Gertow,[‡,■] Steve E. Humphries,[⊥,■] Rainer Rauramaa,[#,■] Ulf de Faire,[▽,▼,■] Andries J. Smit,[○,■] Philippe Giral,[◆,■] Sudhir Kurl,[¶,■] Elmo Mannarino,[+,■] Elena Tremoli,[§,∥,■] Angela Silveira,[‡] John Örvik,[‡] Anders Hamsten,[*,‡] and Craig E. Wheelock[*,†]

[†]Division of Physiological Chemistry 2, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, 17177 Stockholm, Sweden

[‡]Cardiovascular Medicine Unit, Department of Medicine Solna, Center for Molecular Medicine, Karolinska Institutet and Department of Cardiology, Karolinska University Hospital Solna, 17176 Stockholm, Sweden

[§]Department of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano, 20133 Milan, Italy

[∥]Centro Cardiologico Monzino, I.R.C.C.S., 20138 Milan, Italy

[⊥]Cardiovascular Genetics, Institute of Cardiovascular Science, University College of London, London WC1E 6BT, United Kingdom

[#]Foundation for Research in Health Exercise and Nutrition, Kuopio Research Institute of Exercise Medicine, 70100 Kuopio, Finland

[▽]Department of Cardiology, Karolinska University Hospital Solna, 17176 Stockholm, Sweden

[▼]Division of Cardiovascular Epidemiology, Institute of Environmental Medicine, Karolinska Institutet, 17177 Stockholm, Sweden

[○]Department of Medicine, University Medical Center Groningen, 9713 GZ Groningen, The Netherlands

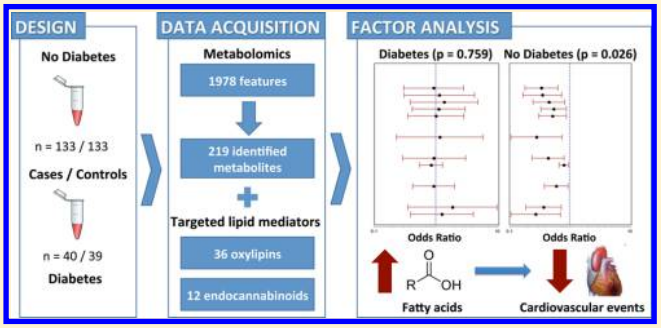
[◆]Assistance Publique - Hopitaux de Paris; Service Endocrinologie-Metabolisme, Groupe Hôpitalier Pitie-Salpetriere, Unités de Prévention Cardiovasculaire, 75004 Paris, France

[¶]Institute of Public Health and Clinical Nutrition, University of Eastern Finland, Kuopio Campus, 70211 Kuopio, Finland

[+]Internal Medicine, Angiology and Arteriosclerosis Diseases, Department of Clinical and Experimental Medicine, University of Perugia, Perugia 06132, Italy

Supporting Information

ABSTRACT: Cardiovascular disease (CVD) is a major cause of morbidity and mortality worldwide, particularly in individuals with diabetes. The current study objective was to determine the circulating metabolite profiles associated with the risk of future cardiovascular events, with emphasis on diabetes status. Nontargeted metabolomics analysis was performed by LC−HRMS in combination with targeted quantification of eicosanoids and endocannabinoids. Plasma from 375 individuals from the IMPROVE pan-European cohort was included in a case-control study design. Following data processing, the three metabolite data sets were concatenated to produce a single data set of 267 identified metabolites. Factor analysis identified six factors that described 26.6% of the variability in the given set of predictors. An association with cardiovascular events was only observed for one factor following adjustment (p = 0.026). From this factor, we identified a free fatty acid signature (n = 10 lipids, including saturated, monounsaturated, and polyunsaturated fatty acids) that was associated with lower risk of future cardiovascular events in nondiabetics only (OR = 0.65, 0.27−0.80 95% CI, p = 0.030), whereas no association was observed among diabetic individuals. These observations support the hypothesis that increased levels of circulating omega-6 and omega-3 fatty acids are associated with protective effects against future cardiovascular events. However, these effects were only observed in the nondiabetic population, further highlighting the need for patient stratification in clinical investigations.



KEYWORDS: *cardiovascular disease, diabetes, fatty acids, metabolomics, eicosanoids, endocannabinoids*

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INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide[1] and is especially pronounced among individuals with diabetes.[2] While multiple potential markers have been proposed for predicting future cardiovascular events, there is significant uncertainty regarding their ability to accurately predict risk.[3] Metabolomics has been successfully applied to determine the circulating metabolic profile in an effort to link specific metabolites to the onset of CVD[4−8] and diabetes,[9−11] as reviewed by Ruiz-Canela et al.[12] and Guasch-Ferré et al.[13] A recent large prospective study of three population-based cohorts employed high-throughput NMR-based metabolomics in combination with a targeted metabolomics platform to identify (sets of) biomarkers that improved CVD risk prediction.[8] In the current study, we applied nontargeted high-resolution mass spectrometry to a pan-European study of cardiovascular disease (Carotid Intima Media Thickness [IMT] and IMT-Progression as Predictors of Vascular Events in a High Risk European Population; IMPROVE[14]). The study objective was to determine the metabolite profiles associated with the risk of future cardiovascular events. These metabolomics studies were complemented with targeted analyses of eicosanoids and endocannabinoids, which have known roles in CVD as well as diabetes.[15−17] We stratified the study population by diabetic status given the importance of the reported biomarkers within the framework of the SUrrogate markers for Micro- and Macro-vascular hard end points for Innovative diabetes Tools (SUMMIT) consortium,[18] which aimed to identify and characterize biomarkers for complications of diabetes.

MATERIALS AND METHODS

Detailed methods are available in the Supporting Information.

Study Population

The current study was based upon the original IMPROVE cohort, which is a multicenter longitudinal cohort study of 3711 subjects designed to identify the main determinants of cIMT in high-risk individuals.[19] Between 2002 and 2004, men and women aged from 55 to 79 years with at least three vascular risk factors (VRFs) but with no symptoms of cardiovascular disease were recruited in seven centers in five European countries (Finland, Sweden, The Netherlands, France, and Italy) and followed for about 3 years. Individuals were considered to possess a VRF when one of the following criteria was satisfied: male sex or at least 5 years after menopause for women; hypercholesterolemia (mean calculated LDL-C blood levels >160 mg/dL or treatment with lipid lowering drugs); hypertriglyceridemia (triglycerides levels >200 mg/dL after diet or treatment with triglycerides lowering drugs); hypoalphalipoproteinemia (HDL-C < 40 mg/dL); hypertension (diastolic blood pressure, DBP > 90 mmHg and/or systolic blood pressure, SBP > 140 mmHg or treatment with antihypertensive drugs); diabetes or impaired fasting glucose (blood glucose level >110 mg/dL or treatment with insulin or oral hypoglycaemic drugs); smoking habits (at least 10 cigarettes/day for at least 30 months); and family history of cardiovascular diseases. The IMPROVE study exclusion criteria were: age under 55 or over 79 years; abnormal anatomical configuration of neck and muscles; marked tortuosity and/or depth of the carotid vessels and/or uncommon location of arterial branches; personal history of myocardial infarction, angina pectoris, stroke, transient ischemic attack, aortic aneurysm, or claudication; revascularization in carotid, coronary, or peripheral arteries, congestive heart failure (III−IV NYHA Class); and history of serious medical conditions that might limit longevity.

During the study span, 215 cardiovascular events were recorded, including myocardial infarction, sudden cardiac death, angina pectoris, ischemic stroke, transient ischemic attack, new diagnosis of intermittent claudication, heart failure, or any surgical intervention or revascularization of coronary or peripheral arteries. The case-control matching yielded 201 pairs, after excluding subjects for whom a mismatch was observed between the diabetes status in the database and the pre-established criteria used to define diabetes in the present report (diagnosis of diabetes and/or treatment with insulin or other hypoglycemic drug, and/or fasting glucose ≥7 mmol/L at the baseline examination). The final cohort for metabolomics analysis included 173 incident cases and 172 controls matched for recruitment center, age, sex, diabetes status, insulin use, statin use, and smoking (Figure 1). From each of these individuals, blood sampling was

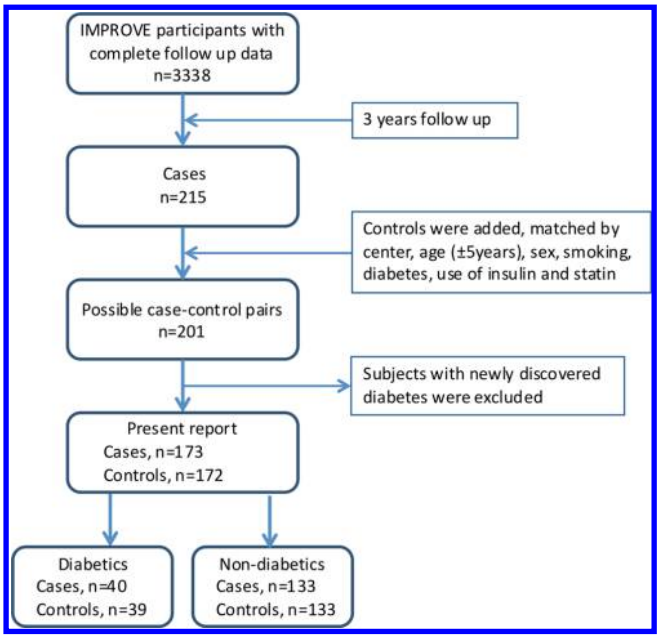

Figure 1. Study design with participant inclusion and matching criteria. Cases were subjects who suffered a cardiovascular event (myocardial infarction, sudden cardiac death, angina pectoris, ischemic stroke, transient ischemic attack, new diagnosis of intermittent claudication, heart failure, or any surgical intervention or revascularization of coronary or peripheral arteries) during the follow up period.

performed after an overnight fast. EDTA plasma samples were prepared and kept frozen at −80 °C until used for centralized laboratory analyses (at the Karolinska Institutet, Sweden).[19] Ethics committee approvals for the study were obtained in each of the seven recruiting centers, and the study followed the respective institutional guidelines. Written informed consents were obtained from all participants. Informed consent for the IMPROVE study includes assessments of CVD risks based on blood samplings for later analyses of blood-based risk markers and genetic variants as well as ultrasound investigations of the carotids.

Metabolomics Analysis

Plasma samples were analyzed using liquid chromatography coupled to high-resolution mass spectrometry (LC−HRMS) as previously published,[20] and described in the Supporting Information. In brief, EDTA plasma samples (400 μL) were thawed on ice and split into three different extraction methods. For reversed-phase metabolomics analysis, 50 μL of plasma was protein-precipitated

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with 3:1 volumes of prechilled methanol. For hydrophilic interaction liquid chromatography (HILIC) metabolomics analysis, 50 μL of plasma were protein-precipitated with 4:1 volumes of acetonitrile. Samples were vortexed and centrifuged, and the supernatant was transferred and stored at −80 °C until the day of analysis. For lipid mediator analysis, solid-phase extraction (SPE) was used to extract lipid mediators from 250 μL of plasma, as previously published.[21,22] Eicosanoids and endocannabinoids were extracted with Waters Oasis HLB (60 mg) SPE cartridges, eluted, and extracts were dried and stored at −80 °C until the day of analysis.

For metabolomics, samples were analyzed using an Ultimate 3000 UHPLC and a Q-Exactive Orbitrap mass spectrometer (ThermoFisher, Waltham, MA). For reversed-phase chromatography, 20 μL of sample was injected on a Thermo Accucore aQ RP C18 column (150 × 2.1 mm, 2.7 μm particle size) and analyzed as described in the Supporting Information. For HILIC chromatography, 12.5 μL of sample was injected on a Merck-Sequant ZIC-HILIC column (150 × 4.6 mm, 3.5 μm particle size) fitted with a Merck Sequant ZIC-HILIC guard column (20 × 2.1 mm) and analyzed as described in the Supporting Information. Mass-spectrometry data were acquired (full-scan mode) in both positive and negative ionization modes (an independent run for each polarity), with a resolution of 70 000 at 200 m/z.

Eicosanoid[21] and endocannabinoid[22] separation were performed as previously published using an Acquity UPLC and a XEVO-TQS triple quadrupole (Waters, Milford, MA) with some modifications. In brief, eicosanoid and endocannabinoid separation were separately performed using an ACQUITY UPLC BEH (Ethylene Bridged Hybrid) C18 column (130 Å, 1.7 μm, 2.1 mm × 150 mm) equipped with a precolumn (ACQUITY UPLC BEH C18 VanGuard precolumn, 130 Å, 1.7 μm, 2.1 mm × 5 mm) as described in the Supporting Information.

Data Processing

MSconvert was used to convert and centroid the raw files to mzXML data format.[23] All chromatograms were evaluated using the open-source software package XCMS[24] performed under the R software environment. Two preliminary approaches were followed for metabolite annotation. Accurate mass and retention time (AMRT) approach was used to compare the physical parameters of accurate mass and retention time of authentic reference standards to those obtained in the metabolomics analysis. The second approach was a putative annotation (AM) based on matching the m/z signals obtained from the metabolomics analysis with those of entries in the human metabolome database (HMDB).[25] Metabolites of interest identified by factor analysis were later subjected to an MS/MS experiment (on the pooled QC sample). The method of identification for each metabolite is reported in Table S1.

Each sample was subjected to analysis in six different, but overlapping methods. To avoid the redundant reporting of the same metabolic signals, an in-house script (VBA-Excel) was used to filter metabolites reported in more than one method. The method of choice was the method with the least analytical variance judged by the coefficient of variance for the QC samples. After imputation of zero values with half the minimum recorded intensity, all metabolomics and background data were combined in one data set for statistical analysis.

Statistical Analysis

The computer software STATA version 11.2 (StataCorp LP, College Station, TX) was used to conduct statistical analysis. Cluster analysis was performed in RStudio (Version 0.98.1062, RStudio, Boston, MA). Forest plots were created using Forest Plot Viewer (SRA International, Durham, NC). Baseline characteristics were reported as median (interquartile range) for continuous and as count (%) for binary variables. Fisher's exact test for parametric data and the Mann−Whitney U test for nonparametric data were used for comparison between two groups. Nonparametric data were log-transformed prior to factor analysis or regression. A two-sided p-value of 0.05 was considered significant. Principal component analysis (PCA) was performed on the univariate (UV)-scaled log10 of the data using SIMCA v14.0 (MKS Umetrics, Sweden).

Metabolomics measurements contained two types of missing data, which were treated differently. Values below the lowest calibration point were replaced with half the limit of detection (LOD) for that metabolite. True missing values appeared if peaks were missing for a reason of analytical failure. Metabolites with >25% of values below the LOD were excluded from the analysis.

To identify a set of uncorrelated factors we performed factor analysis with varimax (orthogonal) rotation. The purpose of factor analysis is to reduce the number of latent variables (or dimensions), which can explain the common variance and correlation of a larger set of original variables. Factor analysis allows identification of factors that account for inter-relationships between these variables.[26] The rationale for rotation is to maximize factor loadings for selected factors while keeping the total variability described by the combination of these factors. Scree plot for eigenvalues versus components was examined to determine the number of factors to retain (Figure S1). The number of factors to be included in the analysis corresponded to the "elbow" in the scree plot. On the basis of a common rule of thumb, significant factor loading is considered to be >0.4, which was therefore the cutoff set for factor loadings.[26,27] Clustering of metabolites was performed using hierarchical clustering (Euclidean distances with Ward's method). In principle, Ward's method estimates the distance between two clusters measured by ANOVA sum of squares and joins clusters to maximize the likelihood, so-called minimum variance method.

Association between these factors and incident cardiovascular events was first assessed by conditional logistic regression analysis for matched pairs in univariate model and with further adjustment for age, body mass index (BMI), antihypertension treatment (HT), high-density lipoprotein (HDL) cholesterol, and antiplatelet medication. Association between individual components and incident cardiovascular events was also analyzed in conditional logistic regression, and results were presented in the form of a forest plot. To evaluate the relationship between the significant factor and its components with time to incident cardiovascular events, we used Cox proportional hazard regression with adjustment for age, gender, and population substructure (assessed by multidimensional scaling 1, MSD1). Hazard ratios were presented per one standard deviation of the predictor. All regression models were stratified by diabetes status. The reported p-values for the metabolites identified by factor analysis were not corrected for multiple hypothesis testing because they are highly correlated metabolites identified in a single factor analysis.

The ability of Factor 1 and individual metabolites within this factor to predict short-term progression of asymptomatic atherosclerosis was assessed by linear regression to change over time in cIMT (cIMT progression) and interadventitia common carotid artery diameter (ICCAD), where ICCAD was measured in plaque-free areas. Progression was an estimate of change over follow-up time assessed by linear regression using measurements obtained

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Table 1. Basic Characteristics of the Study Participants[a]

	diabetics		nondiabetics	
	cases	controls	cases	controls
N	40	39	133	133
age, years[b]	66.5 (61.5−68.4)	65.5 (61.2−68.8)	65.5 (60.4−68.1)	65.2 (60.2−67.5)
sex (F/M), *n* (% of females)[b]	15/25, (38)	16/23, (41)	51/82, (38)	51/82, (38)
BMI, kg/m^2	29.3 (26.9−32.2)	28.1 (24.8−32.4)	26.4 (24.1−29.3)	27.0 (24.4−29.3)
waist−hip ratio	0.96 (0.92−1.00)	0.95 (0.90−1.01)	0.94 (0.87−0.98)	0.93 (0.88−0.97)
waist, cm	101 (97−113)	98 (91−111)	95 (86−100)	96 (87−102)
SBP, mmHg	142 (133−153)	146 (136−160)	144 (131−156)	144 (130−160)
DBP, mmHg	83 (77−87)	85 (81−88)	82 (76−90)	83 (77−90)
cholesterol, mmol/L	4.98 (4.49−5.77)	4.93 (4.54−5.75)	5.66 (5.01−6.41)	5.49 (4.94−6.31)
LDL cholesterol, mmol/L	3.06 (2.63−3.72)	3.06 (2.57−3.71)	3.78 (3.05−4.50)	3.51 (2.79−4.21)
HDL cholesterol, mmol/L	1.05 (0.93−1.27)	1.03 (0.91−1.27)	1.18 (1.04−1.44)	1.21 (1.03−1.51)
triglycerides, mmol/L	1.94 (1.31−2.58)	1.52 (1.09−2.01)	1.39 (1.04−1.93)	1.44 (0.97−2.16)
glucose, mmol/L	7.56 (6.61−9.05)	7.50 (6.65−9.75)	5.40 (4.87−5.75)	5.40 (4.90−5.90)
CRP, mg/mL	2.12 (1.20−4.23)	2.01 (0.91−3.42)	2.19 (0.97−4.42)	1.66 (0.87−3.22)
smoking, *n* (%)[b]	7 (17.5)	8 (20.5)	27 (20.5)	27 (20.5)
pack years, number	15 (0−30)	0 (0−18)	0 (0−17)	0 (0−18)
diseases				
diabetes, *n* (%)	40 (100)	39 (100)	−	−
hypercholesterolemia, *n* (%)	24 (60.0)	23 (60.5)	92 (69.7)	91 (68.4)
hypertriglyceridemia, *n* (%)	17 (42.5)	9 (23.1)	30 (22.7)	32 (24.1)
hypoalphalipoproteinemia, *n* (%)	8 (20.0)	5 (12.8)	22 (16.7)	12 (9.0)
hypertension, *n* (%)	36 (90.0)	32 (82.1)	108 (81.8)	105 (79.0)
medication, *n* (%)	40 (100.0)	35 (89.7)	113 (85.6)	107 (80.5)
glucose-lowering, *n* (%)	29 (72.5)	25 (64.1)	0	0
insulin, *n* (%)	5 (12.5)	5 (12.8)	0	0
lipid lowering, *n* (%)	20 (50.0)	18 (46.2)	55 (42.6)	57 (43.2)
statin, *n* (%)	18 (45.0)	17 (43.6)	46 (34.9)	47 (35.3)
antihypertension, *n* (%)	29 (72.5)	22 (56.4)	74 (56.1)	73 (54.9)
antiplatelet, *n* (%)	17 (42.5)	7 (18.0)	30 (22.7)	19 (14.3)
events	43		145	
cardiac	28 (65.1)	−	77 (53.1)	−
cerebro-vascular	10 (35.7)	−	50 (34.5)	−
peripheral	5 (11.1)	−	18 (12.4)	−

[a]F, females; M, males; BMI, body-mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoprotein; HDL, high density lipoprotein; CRP, C-reactive protein. Smoking refers to ever smoker versus nonsmokers. Lipid-lowering medication includes statin, fibrate and resin. Also, four cases (two diabetics, two nondiabetics) and one nondiabetic control used fish oil. Total event number is higher than number of cases due to multiple events in some of the cases. [b]Matching variables.

at baseline and after 15 and 30 months.[28] The linear regression was stratified by diabetes status and adjusted for age, gender, and corresponding baseline measurement of the carotid artery segment. For Factor 1, an extended model also included MDS1, presence of hypertension, blood glucose, ever smoking, lipid-lowering medication, antiplatelets use, and angiotensin receptor blockers use.

RESULTS

The baseline characteristics of the study population according to the presence of diabetes are summarized in Table 1. Among both diabetics and nondiabetics, the cases showed less favorable anthropometric and metabolic profiles and used more medication compared with the controls. Case-control differences were accentuated among the individuals with diabetes.

Following data processing, the nontargeted metabolomics profiling yielded 1978 unique features, of which 270 were matched to chemical reference standards by accurate mass and retention time. Of the 270 metabolites, 51 had >25% of the values below the limit of detection. These compounds were excluded to give 219 identified metabolites. The remaining 1708 features were annotated putatively. A total of 104 lipid mediators from the eicosanoid and endocannabinoid platforms were screened, of which 48 compounds were present above the limit of quantification. These lipid mediators are generally present at concentrations too low to be detected by metabolomics and were therefore quantified using targeted methods. The metabolomics and lipid mediator data sets were concatenated to produce a single data set of 267 metabolites included in the analyses described below. The full list of reported metabolites is provided in Tables S1−S3. The potential for collection center bias was examined via PCA analysis (Figure S2). No distinguishable clusters were observed on a collection center basis (Figure S2A). However, samples collected in the Nordic countries were distinct from the rest of Europe (Figure S2B). Accordingly, analyses were matched by center to control for potential bias.

The metabolite levels were compared between cases and controls, and no significant patterns were observed in the 267 reported metabolites (Table S4). Accordingly, the data were further analyzed by factor analysis. Analysis of the scree plot showed that six factors had eigenvalues greater than the average eigenvalue (Figure S1). Accordingly, six factors were retained

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Table 2. Prediction of Cardiovascular Events Estimated for Individual Factors

	crude diabetics			crude nondiabetics			adjusted diabetics[b]			adjusted nondiabetics		
factor[a]	OR	95% CI	*p*-value	OR	95% CI	*p*-value	OR	95% CI	*p*-value	OR	95% CI	*p*-value
F1	0.69	0.30−1.64	0.404	0.66	0.46−0.96	0.029	0.86	0.32−2.20	0.759	0.64	0.43−0.95	0.026
F2	1.39	0.25−7.82	0.711	0.65	0.27−1.57	0.341	1.22	0.13−9.35	0.851	0.62	0.23−1.50	0.309
F3	0.36	0.11−1−15	0.085	0.58	0.35−0.95	0.031	0.36	0.06−1.08	0.108	0.63	0.38−1.09	0.087
F4	0.51	0.09−2.85	0.446	0.58	0.31−1.06	0.076	0.50	0.08−5.51	0.495	0.65	0.33−1.20	0.179
F5	0.86	0.51−1.46	0.578	0.79	0.59−1.06	0.122	0.93	0.31−1.39	0.815	0.81	0.58−1.10	0.195
F6	0.93	0.40−1.49	0.653	0.77	0.68−1.28	0.443	0.78	0.33−1.60	0.519	0.96	0.69−1.34	0.787

[a]F1−F6 are derived from factor analysis of the concatenated metabolomics and lipid mediator data sets. OR, odds ratio; 95% CI, 95% confidence interval. [b]Adjustment for age, body-mass index, hypertension, HDL cholesterol, and antiplatelet medication was introduced in the regression model for each factor.

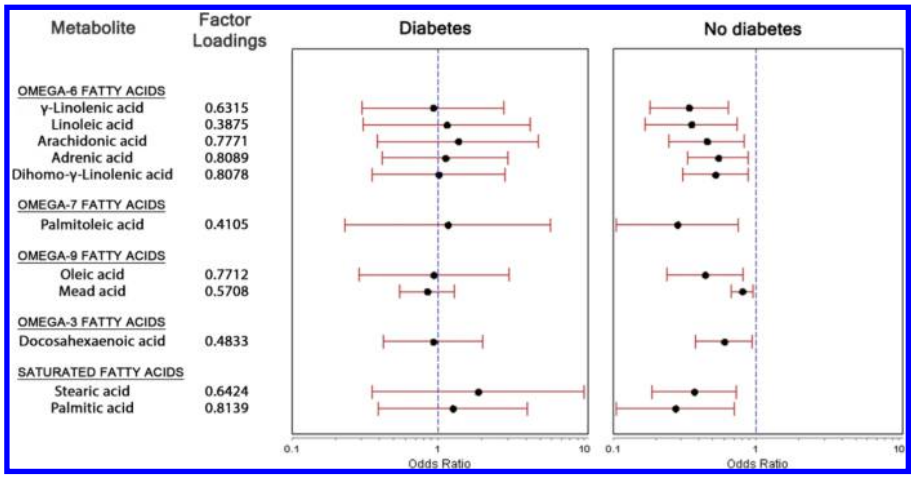

Figure 2. Association of the free fatty acids within Factor 1 with incidence of cardiovascular events stratified by diabetes status. In nondiabetics, high concentrations of fatty acids were associated with lower odds to suffer cardiovascular events (OR = 0.65, 0.27−0.80 95% CI, $p = 0.030$). Linoleic acid was manually added given its relevance to cardiovascular disease, even though the loadings were below the <0.4 cutoff.

Table 3. Relationships of Free Fatty Acids within Factor 1 to Future Cardiovascular Events

	diabetics			nondiabetics		
	HR[a]	95% CI	*p*-value	HR	95% CI	*p*-value
factor 1	1.01	0.61−1.67	0.966	0.80	0.65−0.97	**0.024**
omega-3 fatty acids						
docosahexaenoic acid	1.04	0.70−1.53	0.85	0.86	0.72−1.02	0.085
omega-6 fatty acids						
γ-linolenic acid	1.01	0.65−1.58	0.957	0.72	0.59−0.88	**0.001**
linoleic acid	0.87	0.58−1.29	0.481	0.83	0.69−1.00	0.053
arachidonic acid	1.05	0.0.69−1.60	0.815	0.83	0.70−0.99	**0.037**
adrenic acid	1.01	0.67−1.52	0.967	0.79	0.66−0.95	**0.012**
dihomo-γ-linolenic acid	0.99	0.65−1.52	0.986	0.81	0.69−0.96	**0.015**
omega-7 fatty acids						
palmitoleic acid	0.77	0.	0.199	0.87	0.73−1.05	0.143
omega-9 fatty acids						
oleic acid	1.03	0.71−1.51	0.841	0.83	0.70−0.98	**0.030**
mead acid	0.82	0.56−1.21	0.316	0.85	0.72−1.01	0.059
saturated fatty acids						
stearic acid	1.02	0.73−1.43	0.891	0.81	0.68−0.96	**0.016**
palmitic acid	1.03	0.73−1.46	0.874	0.79	0.66−0.94	**0.009**

[a]Hazard ratios (HR) are per 1-SD log-transformed metabolite concentration and adjusted for age, sex, geographical latitude, hypertension, ever smoking, and antiplatelet medication. 95% CI, 95% confidence interval.

after the varimax orthogonal rotation for the primary analysis. The six factors described 26.6% of the total variability in the given set of predictors (Table S5). Among the diabetics, there was no significant association between any of the six factors and cardiovascular events (Table 2). In nondiabetics, two of the factors were significantly associated with cardiovascular events at $p < 0.05$. Following adjustment for BMI, hypertension, HDL cholesterol, and antiplatelet medication, only Factor 1 (F1) remained significant ($p = 0.026$) (Table 2). F1 contained 39 metabolites (Figure S3), the majority of which were free fatty acids ($n = 10$, Figure 2) or

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their downstream metabolic products (e.g., eicosanoids [n = 19], endocannabinoids [n = 5]). The free fatty acids in F1 included polyunsaturated (PUFAs), monounsaturated (MUFAs), and saturated fatty acids (SAT) (Figure S4). In nondiabetics, high concentrations of free fatty acids were associated with lower odds to suffer cardiovascular events (OR = 0.65, 0.27−0.80 95% CI, p = 0.030), whereas no association was observed among diabetic individuals (Figure 2). A similar trend was observed in Cox proportional hazard regression for the F1 free fatty acids. F1 associated with longer time to incident cardiovascular events in the nondiabetic group (HR = 0.80, 0.65−0.97 95% CI, p = 0.024) (Table 3). All free fatty acids measured were associated with a protective effect in nondiabetic individuals.

In the nondiabetic group, pairwise Spearman rank correlation analysis between the six selected metabolic factors and cIMT measurements identified an association between Factor 2 and all IMT readouts at baseline as well as baseline ICCAD, which disappeared after adjusting for age, gender, and MDS1. Also, in nondiabetics, a significant inverse correlation was observed between Factor 3 and progression of Bulb-IMT$_{mean}$ (r = −0.23, p = 0.002), which disappeared after further adjustments for cardiovascular risk factors and medication (β = −0.011, p = 0.067). Associations between F1 and cIMT variables were essentially nonsignificant. However, a significant correlation was found between F1 and ICCAD change over time in nondiabetics, tested in linear regression with adjustment for MDS1, cardiovascular risk factors (hypertension, blood glucose, ever smoking), and medication (lipid-lowering drugs, angiotensin II receptor blockers, and antiplatelets) (β = −0.008, p = 0.001) (Table S6). To identify the major effects for association with ICCAD change over time among metabolites included in F1, we ran linear regression analysis for each component (Table 4). The strongest association was observed with linoleic-acid-derived metabolites, including: 12(13)-epoxy octadecanoic acid (EpOME; β = −0.018, p = 0.001), 9- and 13-hydroxyoctadecadienoic acid (9-HODE and 13-HODE; β = −0.017, p = 0.002 and β = −0.018, p = 0.001, respectively), as well as 13-keto-octadecadienoic acid (13-KODE; β = −0.014, p = 0.002). Another group of interesting compounds associated with the dynamics of ICCAD were *N*-stearoyl taurine (β = −0.008, p = 0.001) and *N*-palmitoyl taurine (β = −0.011, p = 0.009).

Table 4. Associations between Individual Components of Factor 1 with Change over Time in Inter-Adventitia Common Carotid Artery Diameter (ICCAD) in Nondiabetics (n = 197)[a]

	β	SE	p
12(13)-EpOME	−0.018	0.005	**0.001**
N-stearoyl taurine	−0.008	0.002	**0.001**
13-HODE	−0.018	0.005	**0.001**
13-KODE	−0.014	0.004	**0.002**
9-HODE	−0.017	0.005	**0.002**
N-palmitoyl taurine	−0.011	0.004	**0.009**
LEA	−0.011	0.005	**0.030**
9(10)-EpOME	−0.010	0.005	**0.045**
9-HOTrE	−0.006	0.003	0.050
γ-linoleic acid	−0.008	0.004	0.055
9-KODE	−0.008	0.004	0.060
15-HETE	−0.008	0.005	0.064
arachidonyl glycine	−0.004	0.002	0.070
AEA	−0.007	0.004	0.073
dihomo-γ-linolenic acid	−0.007	0.004	0.096
palmitic acid	−0.009	0.005	0.099
15-KETE	−0.009	0.005	0.100
stearic acid	−0.011	0.006	0.103
OEA	−0.010	0.006	0.110
11(12)-EpETrE	−0.008	0.005	0.114
C20H36O3_HEDE(s)[b]	−0.003	0.002	0.115
oleic acid	−0.008	0.005	0.136
arachidonic acid	−0.008	0.005	0.147
17-HDoHE	−0.006	0.004	0.147
docosahexaenoic acid	−0.005	0.004	0.211
1-stearoyl-2-arachidonoyl PC	−0.005	0.004	0.239
adrenic acid	−0.004	0.004	0.330
PEA	−0.006	0.007	0.339
C20H32O3_HETE(s)[b]	−0.003	0.003	0.353
arachidonoyl PAF C-16	−0.004	0.005	0.372
5-HETE	−0.003	0.005	0.466
mead acid	0.001	0.002	0.506
EKODE	−0.001	0.002	0.562
DIHOMOLEA	0.002	0.004	0.636
5-KETE	0.001	0.004	0.785
9-KOTrE	−0.001	0.003	0.807
palmitoleic acid	0.001	0.006	0.908
8-HDoHE	<0.001	0.004	0.917
15-HETrE	<0.001	0.004	0.982

[a]Values are from linear regression with adjustment for age, gender, and corresponding baseline values. Metabolite nomenclature is provided in Table S1. SE = standard error. [b]Terminology of C20H36O3_HEDE(s) and C20H32O3_HETE(s) indicates that the reported metabolite is a monohydroxy isomer of either eicosadienoic acid or arachidonic acid, respectively; however, the exact position of the hydroxyl group is undetermined.

DISCUSSION

In the present report, nontargeted metabolomics analyses of plasma from a subset of the IMPROVE cohort identified a signature of free fatty acids associated with lower risk of future cardiovascular events in nondiabetic subjects. These observations corroborate the results recently reported by Würtz et al.[8] in a large prospective discovery cohort of 7256 individuals, replicated in two other cohorts of 2622 and 3563 individuals. The two studies had similar objectives; however, Würtz et al.[8] did not directly examine the effect of diabetes. The IMPROVE study recruited subjects at high-risk of CVD, resulting in a cohort with elderly participants (mean age 64 years) and 30% prevalence of diabetes. On the basis of the high prevalence of diabetes and within the framework of the SUMMIT consortium, our analyses were stratified by diabetes status. We observed that the risk for future cardiovascular events differed significantly between the two strata and that omega-6 fatty acids were significantly associated with lower risk of future cardiovascular events only in nondiabetics. Würtz et al.[8] identified omega-6 fatty acids to be significantly associated with lower risk of future cardiovascular events (HR = 0.89) over 15 years of follow-up in a large population with a lower diabetes prevalence (∼7.8%), which potentially explains the lack of reported diabetes-related differences.

We also found that circulating levels of MUFAs, herein represented by palmitoleic and oleicacids, associated with lower risk of cardiovascular events in nondiabetic individuals. This finding agrees with previous reports in which dietary MUFAs were shown to directly correlate with the circulating levels[29] and with a favorable lipoprotein profile and thus lower risk of CVD.[30,31] By contrast, Würtz et al.[8] reported that increased levels of MUFAs were associated with a slightly higher risk for

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cardiovascular events (HR = 1.17). The protective effect that we observed in relation to increased levels of circulating fatty acids is not restricted to one type of fatty acid but includes PUFAs, MUFAs, and SATs, with both omega-6 and omega-3 fatty acids.

There are limited studies to date that perform metabolomics profiling in association with incident CVD. The disparity in analytical approaches and metabolic coverage in the utilized methods makes it challenging to directly compare studies. However, many of the reported studies have observed that levels of circulating free fatty acids are associated with the incidence of cardiovascular events.[12] The exact fatty acid species as well as the trajectory and magnitude of the shift vary with the reported study. Würtz et al.[8] as well as the current study focused on European populations and observed that increased levels of omega-6 and omega-3 circulating fatty acids are associated with lower risk of future cardiovascular events. In a Chinese population, circulating long-chain omega-3 fatty acids and stearic acid were associated with lower risk of acute myocardial infarction, while arachidonic acid levels were associated with a higher risk.[32,33] Of particular interest to the current study was the observation that inclusion of oxylipin metabolites of arachidonic acid did not affect the observed odds ratios.[32] A detailed metabolomics investigation of a German prospective cohort concluded that metabolites of the arachidonic acid pathway are independently associated with risk of myocardial infarction in healthy adults.[34] Accordingly, the exact putative role of omega-6 and omega-3 derived lipid mediators (e.g., oxylipins, eicosanoids) in future cardiovascular events is unclear. There is subsequently interest in measuring these low abundance lipid mediators when performing metabolomics studies. Unfortunately, most general metabolomics profiling methods, and especially metabolomics kits, do not detect these compounds, highlighting the need for targeted methods in combination with metabolomics approaches. In addition, none of the studies listed above stratified the reported population by diabetic status, which, in light of the current study, may further confound the reported observations.

Although not a primary goal, we also analyzed the relationships between metabolomics factors and carotid artery ultrasound measurements taken in the participants at the baseline and in progression over 3 years of follow-up. In the IMPROVE study, the progression of the maximum IMT detected after 15 months in the whole carotid tree regardless of location (Fastest-IMT$_{(max\text{-}progr)}$) was significantly associated with the risk of subsequent vascular events, whereas none of the other cIMT measures showed predictive value.[35] In the present study, the only significant association found was between F1 and lower change over time in ICCAD in nondiabetics. ICCAD measured in plaque-free areas is assumed to reflect carotid expansion due to atherosclerosis and correlates with several vascular risk factors. Interestingly, the protective associations between F1 and change in ICCAD were driven by metabolic products of linoleic acid (12[13]-EpOME and 9[10]-EpOME, 9-HODE, 13-HODE, and 13-KODE) and taurine derivatives (*N*-stearoyl taurine and *N*-palmitoyl taurine) (Table 4). The findings on linoleic acid (and its derivatives) are unclear, but a recent meta-analysis reported a suggestive relationship between dietary linoleic acid and diabetes as well as CVD.[36] Taurine, an abundant amino-acid-like compound distributed throughout human tissues, has a long list of biological activity including atheroprotective, anti-inflammatory, and antiobesity effects.[37,38] Taurine has even been studied in relation to cardiovascular prevention and obesity, although the effects of taurine ingestion in humans remain unclear.[37,38] The metabolic effects of palmitic and stearic conjugates have not been well studied and are unclear in the current context.

All participants of the present study were Europeans, which, to some extent, precludes generalization of the observations to other populations. Another limitation is the relatively small size, particularly of the diabetes subset, and lack of a replication cohort. The current study does confirm previous similar results in larger populations[8] but suggests that those findings are not applicable to entire populations. In addition, there is a potential bias in the metabolites identified via metabolomics. While the method is comprehensive, there is a possibility of metabolites not detected with the current method being of interest in understanding cardiovascular risk. This potential metabolite bias does not affect the accuracy of the reported results but simply highlights that there may be additional biochemical information on interest. In summary, the lack of protective effects observed for any of the measured fatty acids, with respect to occurrence of cardiovascular events among diabetic participants, calls for further studies into the increased CVD risk in these patients. In addition, these findings highlight the utility of stratifying populations on multiple clinical and physiological factors (e.g., diabetic status, sex, therapeutic response). This type of analysis is an important component of stratified medicine, as demonstrated by the reported observation that the protective effects of circulating fatty acids is only observed in a nondiabetic subgroup, which can have repercussions in study design and statistical analysis as well as the primary study findings.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00671.

Extended methods description and supporting data. Figure S1. Scree plot representing the eigenvalues versus the factor numbers. Figure S2. PCA score plots of all samples included in the study. Figure S3. Forest plot representing association of individual components of Factor 1 in the study population stratified by diabetes status. Figure S4. Dendrogram showing clustering of variables in Factor 1. Table S5. Variable composition of the six individual factors sorted by their loadings within each factor. Table S6. Relationships of Factor 1 to carotid IMT progression and change over time in carotid diameter. (PDF)

Table S1. Metabolite IDs for all reported compounds. Table S2. Measured concentrations of eicosanoids and endocannabinoids. Table S3. Measured abundances of metabolites (arbitrary units). Table S4. Comparison of cases versus controls. (XLSX)

■ AUTHOR INFORMATION

Corresponding Authors

*C.E.W.: E-mail: Craig.Wheelock@ki.se. Tel +46 8 524 876 30.
*A.H.: E-mail: Anders.Hamsten@ki.se. Tel: +46 8 517 732 01.

ORCID

Craig E. Wheelock: 0000-0002-8113-0653

Author Contributions

□M.A.K., O.M., and A.C. contributed equally. M.A.K., O.M., J.Ö., A.H., and C.E.W. conceived the study. Clinical samples were acquired by D.B., F.V., S.E.H., R.R., U.d.F., A.J.S., P.G., S.K., E.M., E.T., and A.H. Metabolomics data were acquired by M.A.K. and A.C. Statistical analysis was performed by M.A.K., O.M., A.C.,

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and J.Ö. Data interpretation was performed by M.A.K., O.M., A.C., K.G., A.S., J.Ö., A.H., and C.E.W. O.M., A.C., A.S., and C.E.W. prepared the tables and figures. The manuscript was written by O.M., A.C., A.S., J.Ö., and C.E.W. All authors reviewed the final draft of the manuscript and gave final approval of the version to be published.

Notes

The authors declare no competing financial interest.
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■ ABBREVIATIONS

CVD, cardiovascular diseases; CV, coefficient of variance; EpOME, epoxy octadecanoic acid; HODE, hydroxyoctadecadienoic acid; HRMS, high-resolution mass spectrometry; HILIC, hydrophilic interaction liquid chromatography; HMDB, human metabolome database; HR, hazard ratio; ICCAD, interadventitia common carotid artery diameter; IMT, intima media thickness; IMPROVE, IMT-Progression as Predictors of Vascular Events in a High Risk European Population; KODE, keto-octadecadienoic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; QC, quality control; RP, reversed-phase; RT, retention time; SAT, saturated fatty acid; SUMMIT, SUrrogate markers for Micro- and Macro-vascular hard end points for Innovative diabetes Tools consortium

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