International Journal of Epidemiology, 2018, 1–16
doi: 10.1093/ije/dyy198
Original article

Original article

Lipid metabolic networks, Mediterranean diet and cardiovascular disease in the PREDIMED trial

Dong D Wang,[1] Yan Zheng,[1] Estefanía Toledo,[2,3,4] Cristina Razquin,[2,3,4] Miguel Ruiz-Canela,[2,3,4] Marta Guasch-Ferré,[1,5] Edward Yu,[1] Dolores Corella,[4,6] Enrique Gómez-Gracia,[7] Miquel Fiol,[4,8] Ramón Estruch,[4,9] Emilio Ros,[4,10] José Lapetra,[4,11] Montserrat Fito,[4,12] Fernando Aros,[4,13] Lluis Serra-Majem,[4,14] Clary B Clish,[15] Jordi Salas-Salvadó,[4,5] Liming Liang,[16,17] Miguel A Martínez-González[1,2,3,4] and Frank B Hu[1,17,18]*

[1]Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, MA, USA, [2]Department of Preventive Medicine and Public Health, University of Navarra, Pamplona, Spain, [3]IDISNA (Instituto de Investigación Sanitaria de Navarra), Pamplona, Spain, [4]CIBER Fisiopatología de la Obesidad y Nutrición (CIBERObn), Instituto de Salud Carlos III, Madrid, Spain, [5]Human Nutrition Unit, Faculty of Medicine and Health Sciences, Institut d'Investigació Sanitària Pere Virgili, Rovira i Virgili University, Reus, Spain, [6]Department of Preventive Medicine, University of Valencia, Valencia, Spain, [7]Department of Preventive Medicine, University of Málaga, Málaga, Spain, [8]Institute of Health Sciences IUNICS, University of Balearic Islands and Hospital Son Espases, Palma de Mallorca, Spain, [9]Department of Internal Medicine, Institut d'Investigacions Biomediques August Pi Sunyer (IDI- BAPS), University of Barcelona, Barcelona, Spain, [10]Lipid Clinic, Department of Endocrinology and Nutrition, Institut d'Investigacions Biomediques August Pi Sunyer (IDI- BAPS), University of Barcelona, Barcelona, Spain, [11]Department of Family Medicine, Primary Care Division of Sevilla, San Pablo Health Center, Sevilla, Spain, [12]Cardiovascular and Nutrition Research Group, Institut de Recerca Hospital del Mar, Barcelona, Spain, [13]Department of Cardiology, University Hospital of Alava, Vitoria, Spain, [14]Department of Clinical Sciences, University of Las Palmas de Gran Canaria, Las Palmas, Spain, [15]Broad Institute and MIT, Harvard University, Cambridge, MA, USA, [16]Department of Epidemiology, [17]Department of Biostatistics, Harvard T.H. Chan School of Public Health, Boston, MA, USA and [18]Channing Division for Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, MA, USA

*Corresponding author. Departments of Nutrition and Epidemiology, Harvard T.H. Chan School of Public Health, 665 Huntington Avenue, Boston, MA 02115, USA. E-mail: nhbfh@channing.harvard.edu

Editorial decision 20 August 2018; Accepted 10 September 2018

Abstract

Background: Perturbed lipid metabolic pathways may play important roles in the development of cardiovascular disease (CVD). However, existing epidemiological studies have focused more on discovering individual lipid metabolites for CVD risk prediction rather than assessing metabolic pathways.

Methods: This study included a subcohort of 787 participants and all 230 incident CVD cases from the PREDIMED trial. Applying a network-based analytical method, we identified lipid subnetworks and clusters from a global network of 200 lipid metabolites and linked these subnetworks/clusters to CVD risk.
Results: Lipid metabolites with more double bonds clustered within one subnetwork, whereas lipid metabolites with fewer double bonds clustered within other subnetworks. We identified 10 lipid clusters that were divergently associated with CVD risk. The hazard ratios [HRs, 95% confidence interval (CI)] of CVD per a 1-standard deviation (SD) increment in cluster score were 1.39 (1.17–1.66) for the hydroxylated phosphatidylcholine (HPC) cluster and 1.24 (1.11–1.37) for a cluster that included diglycerides and a monoglyceride with stearic acyl chain. Every 1-SD increase in the score of cluster that included highly unsaturated phospholipids and cholesterol esters was associated with an HR for CVD of 0.81 (95% CI, 0.67–0.98). Despite a suggestion that MedDiet modified the association between a subnetwork that included most lipids with a high degree of unsaturation and CVD, changes in lipid subnetworks/clusters during the first-year follow-up were not significantly different between intervention groups.
Conclusions: The degree of unsaturation was a major determinant of the architecture of lipid metabolic network. Lipid clusters that strongly predicted CVD risk, such as the HPC cluster, warrant further functional investigations.

Key words: Lipid network, Mediterranean diet, cardiovascular disease

Key Messages

- We applied a network-based analytical approach to construct a global network of 200 plasma lipid metabolites, and found many lipid subnetworks/clusters that may indicate perturbed pathways involved in the pathogenesis of CVD in a subpopulation of the PREvencion con Dieta MEDiterranea (PREDIMED) trial.
- By incorporating the structural information of lipid network into the framework of regression, we found divergent associations of the lipid subnetworks/clusters with the incidence of cardiovascular disease.
- Several lipid clusters, such as the hydroxylated phosphatidylcholine pathway and a pathway including diglycerides and a monoglyceride with stearic acyl chain, strongly predicted the risk of cardiovascular disease.

Introduction

Hundreds of thousands of structurally diverse lipids play important roles in cellular signalling and energy storage as well as the structure of biological membranes, and may be involved in the pathological alterations of cardiovascular disease (CVD).[1] However, our current understanding of the role of lipids in the pathogenesis of CVD is mainly confined to broad classes of plasma lipid markers, such as total triglycerides (TG) and low-density lipoprotein cholesterol (LDL-C), rather than individual lipid molecules. Lipidomics provide powerful and high-throughput tools to obtain structural details of a specific lipid metabolite, such as the polar head group, the covalent nature of the linkage with fatty acid chains, the length of the aliphatic chain of fatty acid and the amount of double bonds. Several recent studies have used lipidomics for risk prediction of CVD and found divergent associations of lipid metabolites, partially explained by their fatty acyl group composition, with incident CVD, providing an early glimpse into pathological alterations in lipid metabolic pathways that promote CVD.[2–6] Furthermore, some lipids, such as ceramides[7,8] and diacylglycerols (DAGs), may exert potent biological functions, including pro-inflammatory changes and impairment of insulin sensitivity, beyond their fatty acyl group compositions.

Current lipidomics studies[2–6] have focused more on discovering individual lipid metabolites predictive of CVD risk than assessing the perturbed pathways responsible for pathological processes. The common practice of the studies

has mainly relied on a large number of statistically independent tests in conjunction with stringent multiple-comparison corrections, inherently neglected higher order dependencies and pathways among lipid metabolites, and has not generated reproducible findings.[9] However, a matrix of individual molecular entities usually exerts their cellular functions interactively and through a network of biochemical pathways. Furthermore, groups of metabolites involved in the same pathway tend to be highly correlated and associated with disease risk of similar strength and direction. Therefore, the biological pathway may serve as a more appropriate analysing unit to understand the complex biological system.[10] The network-based analytical approach is a powerful tool for detecting clusters of metabolites that may represent biologically meaningful pathways. Furthermore, the network-based approach provides a natural way for dimension reduction in high-dimensional 'omics' datasets. Previous studies have shown that pathways or clusters derived by the network-based approaches corresponded to known biochemical interactions and metabolic pathways.[11–15] For example, Krumsiek *et al.* recovered various modules from a network of plasma metabolites that represent metabolic pathway reactions, by applying the Gaussian graphical model.[13] Compared with traditional dimension-reduction tools, such as principal component analysis, a major advantage of the network-based approaches is the ability to identify small-scope biologically meaningful pathways, which facilitates the construction of follow-up hypotheses in a functional context.[16,17]

The PREvencion con DIeta MEDiterranea (PREDIMED) trial[18,19] evaluated the effects of overall dietary pattern on the primary prevention of CVD, and found that the Mediterranean diet (MedDiet) enriched with extra-virgin olive oil or mixed nuts significantly reduced CVD events by approximately 30% compared with the control diet (low-fat diet).[20] The 2015–2020 Dietary Guidelines for Americans[21] and the American Heart Association (AHA)[22] both recommend the MedDiet for CVD prevention, based on strong and consistent evidence on a hard CVD endpoint from the PREDIMED trial[20] and prospective cohort studies.[23–26] To strengthen the evidence base of current dietary guidelines and develop more effective dietary prevention strategies, it is critical to investigate the underlying biological mechanisms through which the MedDiet may prevent CVD. Many mechanisms closely related to lipid metabolism, such as reduction of inflammation,[27–31] protection of LDL-C against oxidation,[32,33] enhanced endothelial function,[29,34,35] and lower level of atherogenic lipoproteins,[36] have been proposed to be responsible for the cardiovascular benefits of MedDiet, yet molecular-level evidence is still limited.

The present study applied a network-based analytical approach to lipidomics data in a subpopulation from the PREDIMED trial. We hypothesized that various subsets of lipid metabolic network were divergently associated with the incidence of CVD. We also investigated whether the associations of lipid subnetworks/clusters and CVD varied by the MedDiet interventions, and whether the MedDiet interventions had differential effects on the changes in different lipid metabolic pathways from baseline to the 1-year follow-up.

Methods

Study design and population

This study adopted a case-cohort design[37,38] and consisted of all 230 incident CVD cases diagnosed during up to a 7.4-year follow-up (average follow-up = 4.8 years) and 787 randomly selected participants at baseline (subcohort, 10% of the enrolled participants) in the PREDIMED trial. The subcohort included 37 overlapping cases of CVD. The case-cohort design preserves random intervention assignments and maintains the causal integrity of the randomized design of the trial. The PREDIMED trial [www.predimed.es] was conducted from 2003 through 2010 in 11 centres in Spain, to assess the effects of the MedDiet on the primary prevention of CVD. At baseline, this trial enrolled 7447 participants aged 55–80 years with high cardiovascular risk but free from diagnosed CVD at baseline. Participants were randomly assigned to a MedDiet supplemented with extra-virgin olive oil (MedDiet + EVOO), a MedDiet supplemented with nuts (MedDiet + nuts) or a control diet consisting of advice to reduce the intake of all types of fat. The protocol was approved by the institutional review boards at all study locations, and all participants provided written informed consent. Detailed information about the PREDIMED trial can be found elsewhere.[18,20]

Study samples and lipidomics profiling

All analyses used fasting (fasting for $\geq$8 h) plasma EDTA samples collected at baseline and year 1. All samples were processed at each recruiting centre no later than 2 h after collection and stored in -80°C freezers. Samples from cases and subcohort participants were randomly distributed before being shipped to the Broad Institute in Boston, MA, for lipidomics assays. Plasma lipid metabolites were identified by the length of aliphatic chain of fatty acid and the number of double bonds. Details of the LC-MS platform can be found in Supplementary methods, available as Supplementary data at *IJE* online.

Ascertainment of CVD outcomes

The primary CVD outcome was a composite of non-fatal acute myocardial infarction (AMI), non-fatal stroke or cardiovascular death. Information on outcomes was collected from continuous contact with participants and primary health care physicians, annual follow-up visits, yearly *ad hoc* reviews of medical charts and annual consultation of the National Death Index. Study physicians who were blinded to the intervention collected information on primary outcomes. Blinded to the intervention assignment, the clinical endpoint committee adjudicated the events according to the standard criteria.[39–44]

Measurements of covariates

At baseline and yearly follow-up visits, medical conditions, family history of disease and risk factors were collected through a questionnaire. At baseline and during annual visits, participants completed a 14-item questionnaire in a personal interview with a registered dietitian, to assess their adherence to the MedDiet.[45] At baseline and annually, trained personnel measured participants' body weight, height and blood pressure according to the study protocol. Body mass index (BMI) was calculated as weight in kilograms divided by height in metres squared.

Statistical analysis

We transformed concentrations of lipid metabolites to the natural logarithm scale to render the distributions approximately Gaussian as well as to stabilize the variance. During the network construction step, we first constructed a global network of lipid metabolites based on partial correlations with *P*-values ≤ 0.05 among all 200 targeted lipid metabolites. Each partial correlation between a pair of lipid metabolites was calculated by the Kendall rank correlation analysis conditional on the remaining 198 lipid metabolites.[46] Second, we detected a limited number of subnetworks of the global network by applying a modularity detection algorithm, the Greedy Optimization, that has been previously applied in the gene expression network analysis.[47,48] We named each subnetwork according to the features of the major 'hub' metabolites (i.e. metabolites connected with more neighbour metabolites) within it. Differences across these subnetworks reflected potential biological mechanisms underlying the general structure of global network. Third, we applied the Benjamini-Hochberg (B-H) procedure to all the edges in the initial global network and removed paths between lipid metabolites at the criterion of adjusted *P*-values greater than 0.05. Fourth, we repeated the modularity detection algorithm in the global network after the removal of paths. Notably, we found a large number of small-scope components of the global network. These small-scope components contained lipid metabolites that tended to be closely connected within pathways of lipid metabolism. We named these small-scope components 'lipid clusters' and investigated their associations with CVD risk in the context of potential biological functions of each metabolic pathway.

In the network-guided regression step, we first extracted the information of pathway structure, the topological matrix,[49] to calculate the pathway weight for each lipid metabolite. The topological matrix measures not only the relationship among lipid metabolites based on the pair of lipid metabolites themselves, but also their relationship to all other lipid metabolites in the pathway. Details of the topological matrix can be found elsewhere.[49] Second, using the pathway weights, we calculated the subnetwork/cluster scores as the weighted sum of levels of lipid metabolite within each lipid subnetwork/cluster. Last, we modelled these scores as the exposure variables in the Cox proportional hazards (PH) model. We categorized all the participants into quartiles of the score for lipid subnetwork/cluster, based on their distribution in the subcohort. Person-years of follow-up were calculated from baseline to the earliest CVD event, loss to follow-up or the end of follow-up. Weighted Cox PH models stratified on intervention group assignments (MedDiet + EVOO, MedDiet + nuts, and control) were applied to estimate hazard ratios (HRs) and their 95% confidence intervals (CIs) of CVD risk, comparing participants in each quartile with the lowest quartile of the subnetwork/cluster scores, as well as HRs (95% CIs) associated with a 1-standard deviation (SD) increment in the scores. We used the weighting scheme suggested by Barlow *et al.*[50,51] to account for the over-representation of cases due to the case-cohort design in the weighted Cox PH models. We also performed secondary analyses on AMI and stroke separately. All the models simultaneously adjusted for age, sex, BMI, family history of CVD, smoking status and histories of hypertension, dyslipidaemia and diabetes at baseline. In a sensitivity analysis, the Cox PH models adjusted for levels of plasma lipids [TG, LDL-C and high-density lipoprotein cholesterol (HDL-C)] measured at baseline in addition to the aforementioned covariates except history of dyslipidaemia. To examine the cross-sectional associations of the lipid subnetworks/clusters with plasma lipids measured in baseline plasma samples, we applied general linear models that included the lipid subnetwork/cluster scores as exposures and plasma lipids [TG, total cholesterol (TC), LDL-C and HDL-C] as outcomes.

To examine whether the associations between the lipid subnetworks/clusters and incident CVD varied by the intervention groups, we first calculated the multivariable-adjusted

HRs of CVD separately in the MedDiet and control groups. We then added a multiplicative term between intervention assignment and the score into the multivariable Cox PH models, to test interaction using the likelihood ratio test. We compared the temporal (1-year) changes in the lipid subnetwork/cluster scores between intervention and control groups, using a linear mixed model. The PREDIMED trial recently reported some irregularities in the randomization procedures, which affected a small subset of participants.[20] To address this issue, we additionally adjusted for two propensity scores (built with 30 predictors of intervention allocation)[20] and applied robust estimates of the variance to correct for intracluster correlation in the Cox PH model for testing the interactions between the lipid subnetworks/clusters and intervention groups, and in the linear mixed model for comparing temporal changes in the lipid subnetwork/cluster scores between intervention groups. We also examined the associations of 1-year changes in the lipid subnetwork/cluster scores with the incident CVD cases that occurred from the third year to the end of follow-up. All analyses were performed using R software, version 3.3.2 (R Core Team) and SAS software, version 9.4 (SAS Institute, NC).

Results

Characteristics of study participants and global network of lipid metabolites

The baseline characteristics of the subcohort were very similar to those of the full cohort in the PREDIMED trial[20] (Table 1). We observed high correlations among 200 targeted lipid metabolites measured in plasma samples collected at baseline. The correlations were particularly high among individual lipid metabolites within the same lipid subclass (Supplementary Figure 1, available as Supplementary data at *IJE* online). In the global network of lipid metabolites, each vertex represented a lipid metabolite and each path stood for a significant partial correlation between a pair of lipid metabolites (Supplementary Figure 2, available as Supplementary data at *IJE* online). Among the major subnetworks of lipid metabolites, three subnetworks were partially overlapped. In general, most lipid metabolites with a larger number of double bonds clustered within the same subnetwork (named 'unsaturated subnetwork'), whereas most lipid metabolites with a smaller number of double bonds clustered within the other three subnetworks [named 'saturated phospholipid subnetwork', 'saturated glyceride subnetwork' and 'monoacylglyceride (MAG) subnetwork', Figure 1 and Supplementary Figures 3–6, available as Supplementary data at *IJE* online]. Supplementary Table 1, available as Supplementary data at *IJE* online, shows the lipid metabolites that were included in each subnetwork. Using the ratio of number of double bonds to length of aliphatic chain of fatty acid as a measure of unsaturation for a specific lipid metabolite, we found that the average ratios were 0.07 for the saturated phospholipid subnetwork, 0.15 for the unsaturated subnetwork, 0.06 for the saturated glyceride subnetwork and 0.04 for the MAG subnetwork. The components of the saturated phospholipid subnetwork were diverse; the major 'hub' metabolites included sphingomyelins and cholesterol esters (CE) with saturated fatty acyl chains as well as various phosphatidylcholines (PCs) and phosphatidylcholine plasmalogens with fewer double bonds (Supplementary Figure 3, available as Supplementary data at *IJE* online). Lipid metabolites with unsaturated fatty acyl chains, such as triacylglycerides (TAGs) (58: 11, 56: 7, 58: 8 and 60: 12), CE (20: 5), and PC (40: 10), were the major 'hubs' within the unsaturated subnetwork (Supplementary Figure 4, available as Supplementary data at *IJE* online). The major components of the saturated glyceride subnetwork were TAGs and DAGs with fewer double bonds (Supplementary Figure 5, available as Supplementary data at *IJE* online). The MAG subnetwork with DAG (36: 0) as the 'hub' metabolite was relatively small and included six lipid metabolites (Supplementary Figure 6, available as Supplementary data at *IJE* online).

Table 1 shows that participants with a higher score for the saturated phospholipid subnetwork had a higher prevalence of hypertension ($P_{trend} = 0.03$), but lower prevalence of diabetes and current smoking (both $P_{trend} < 0.001$) at baseline. The score for the unsaturated subnetwork was inversely associated with age ($P_{trend} < 0.001$) and prevalence of diabetes ($P_{trend} = 0.02$), but positively associated with the prevalence of dyslipidaemia ($P_{trend} < 0.001$). Participants with a higher score of the saturated glyceride subnetwork had higher BMI ($P_{trend} < 0.001$) and prevalence of diabetes ($P_{trend} = 0.01$). Participants with a higher score of the MAG subnetwork had a non-significantly higher score that measured the adherence to MedDiet.

Major lipid subnetworks and CVD

The four major subnetworks were associated with the incidence of CVD in divergent directions (Table 2). The HR of CVD comparing extreme quartiles of the score for the unsaturated subnetwork was 0.62 (95% CI, 0.39–1.00, $P_{trend} = 0.007$) after adjusting for age, sex, BMI, family history of CVD and smoking status. The association was attenuated after further adjustment for baseline hypertension, dyslipidaemia and diabetes but remained significant; every 1-SD increment in the score for the unsaturated subnetwork was associated with a 19% decrease in the risk of CVD (HR = 0.81, 95% CI, 0.67–0.98). The scores for the saturated phospholipid subnetwork and saturated glyceride subnetwork were each positively associated with

Table 1. Baseline characteristics of study participants

	Cases	Subcohort[a]	*P*-value[b]	Quartiles of major subnetwork score[d]										
				Saturated phospholipid subnetwork		P_{trend}[c]	Unsaturated subnetwork		P_{trend}	Saturated glyceride subnetwork		P_{trend}	Monoglyceride subnetwork		P_{trend}
	($n=230$)	($n=787$)		Q1	Q4		Q1	Q4		Q1	Q4		Q1	Q4[d]	
Intervention group, %															
Control	83 (36.1)	234 (29.7)	0.15	66 (33.7)	56 (28.4)	0.79	60 (30.6)	60 (30.5)	0.42	61 (31.1)	60 (30.5)	0.45	48 (24.5)	61 (31.0)	0.08
MedDiet + EVOO	82 (35.7)	291 (37.0)		62 (31.6)	77 (39.1)		66 (33.7)	78 (39.6)		72 (36.7)	69 (35.0)		79 (40.3)	77 (39.1)	
MedDiet + nuts	65 (28.3)	262 (33.3)		68 (34.7)	64 (32.5)		70 (35.7)	59 (29.9)		63 (32.1)	68 (34.5)		69 (35.2)	59 (29.9)	
Men, %	139 (60.4)	337 (42.8)	<0.001	119 (60.7)	47 (23.9)	<0.001	96 (49.0)	86 (43.7)	0.28	96 (49.0)	78 (39.6)	0.05	93 (47.4)	85 (43.1)	0.31
Family history of premature CHD, %	44 (19.1)	196 (24.9)	0.07	48 (24.5)	55 (27.9)	0.96	52 (26.5)	45 (22.8)	0.73	55 (28.1)	43 (21.8)	0.40	53 (27.0)	45 (22.8)	0.29
Smoking, %			<0.001			<0.001			0.52			0.17			0.52
Never	104 (45.2)	491 (62.4)		102 (52.0)	146 (74.1)		124 (63.3)	123 (62.4)		113 (57.7)	130 (66.0)		119 (60.7)	124 (62.9)	
Current	46 (20.0)	96 (12.2)		30 (15.3)	17 (8.6)		16 (8.2)	30 (15.2)		30 (15.3)	26 (13.2)		22 (11.2)	26 (13.2)	
Former	80 (34.8)	200 (25.4)		64 (32.7)	34 (17.3)		56 (28.6)	44 (22.3)		53 (27.0)	41 (20.8)		55 (28.1)	47 (23.9)	
Hypertension, %	190 (82.6)	659 (83.7)	0.69	157 (80.1)	176 (89.3)	0.03	156 (79.6)	169 (85.8)	0.13	161 (82.1)	166 (84.3)	0.36	158 (80.6)	167 (84.8)	0.49
Dyslipidaemia, %	134 (58.3)	579 (73.6)	<0.001	139 (70.9)	148 (75.1)	0.09	122 (62.2)	162 (82.2)	<0.001	139 (70.9)	153 (77.7)	0.13	148 (75.5)	150 (76.1)	0.76
Diabetes, %	149 (64.8)	372 (47.3)	<0.001	122 (62.2)	72 (36.5)	<0.001	108 (55.1)	84 (42.6)	0.02	83 (42.3)	105 (53.3)	0.01	86 (43.9)	100 (50.8)	0.13
Age (years)	69.5±6.5	67.2±5.9	<0.001	67.6±6.1	66.5±5.8	0.08	68.1±6.0	66.2±5.7	<0.001	67.6±5.7	66.6±6.1	0.08	67.2±5.9	66.3±5.9	0.19
BMI (kg/m^2)	29.6±3.7	29.8±3.6	0.63	30.0±3.5	29.5±3.8	0.23	30.1±4.0	29.7±3.4	0.35	28.8±3.7	30.8±3.7	<0.001	29.8±3.5	29.7±3.6	1.00
Adherence to MedDiet[e]	8.4±1.8	8.8±1.9	0.006	8.8±2.1	8.7±2.0	0.76	8.7±2.0	9.0±1.7	0.06	9.0±1.8	8.6±1.8	0.07	8.7±1.9	9.0±1.9	0.07

Values were *n* (%) for categorical variables and mean ± standard deviation for continuous variables.

[a]The subcohort also included 37 cases.

[b]χ^2 test was used for categorical variables and t test was used for continuous variables.

[c]To quantify a linear trend, we assigned the median within each quartile and modelled this scored trend variable continuously; Wald testing was used for calculating *P* for trend. The logistic model was used for categorical variables and the general linear model was used for continuous variables.

[d]Quartiles were calculated based on the distribution of the major subnetwork score in the subcohort.

[e]Adherence to the Mediterranean diet was assessed by a 14-item dietary screener.

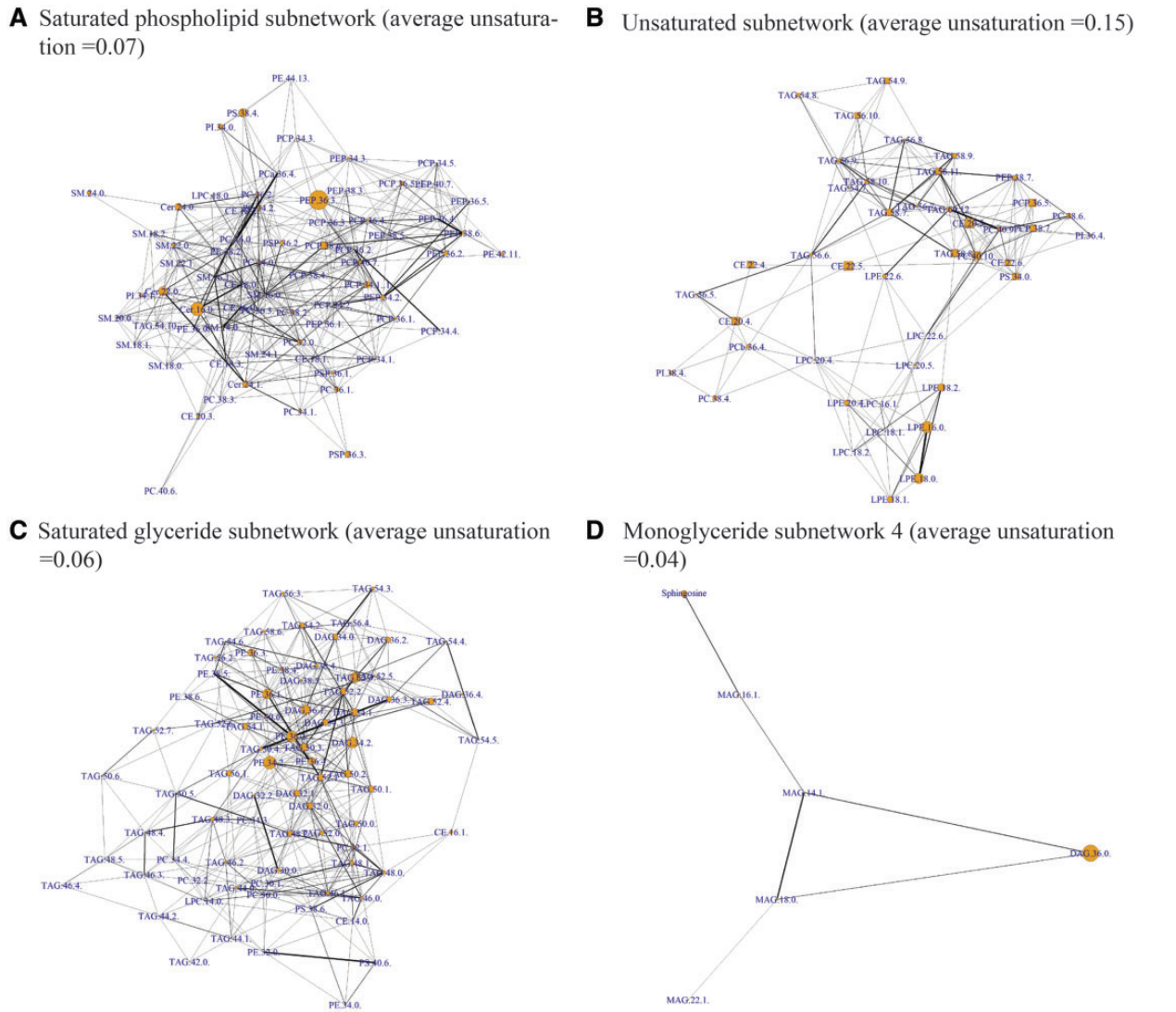

Figure 1. Major lipid subnetworks detected from a global network of 200 plasma lipid metabolites in a subcohort of 787 participants and all 230 incident cases of cardiovascular diseases from the PREDIMED trial.

The digits after the first period stand for number of carbon atoms on the fatty acyl chain. The digits after the second period stand for number of double bonds. Size of vertex is in proportion to −log (*P*-value) of the hazard ratio (HR) for the risk of composite cardiovascular disease endpoint associated with 1-standard deviation increment in the concentration of individual lipid metabolite. Square vertex corresponds to lipid metabolite associated with HR <1.00. Circle vertex corresponds to lipid metabolite with HR ≥ 1.00. Average of unsaturation for each major subnetwork was measured as the average of the ratios of number of double bonds to length of aliphatic chain of fatty acid for specific lipid metabolites with the major subnetwork. Phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylethanolamine plasmalogen (PEP), phosphatidylserine (PS), phosphatidylserine plasmalogen (PSP), phosphatidylinositol (PI), Phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylcholine plasmalogen (PCP), hydroxylated phosphatidylcholine (HPC), cholesterol esters (CE), diacylglycerol (DAG), monoacylglycerol (MAG), triacylglycerol (TAG), sphingomyelins (SM), ceramide (Cer).

CVD risk. The HRs of CVD, comparing extreme quartiles, were 1.76 (95% CI, 1.09-2.86, $P_{trend} = 0.04$) for the score of the saturated phospholipid subnetwork and 1.57 (95% CI, 0.94-2.64, $P_{trend} = 0.04$) for the score of the saturated glyceride subnetwork in the final models. We found a significant positive association between the score of the MAG subnetwork and the incidence of CVD in the model adjusting for major risk factors and confounding factors, but this association became marginally significant in the final model. Secondary analyses on stroke and AMI yielded generally similar associations between baseline subnetwork scores and the specific CVD outcomes, compared with the main analysis of the composite CVD outcome (Supplementary Table 2, available as Supplementary data at *IJE* online).

We found a suggestion of a significant interaction between the MedDiet intervention and the score of the unsaturated subnetwork ($P_{interaction} = 0.04$, Supplementary

Table 2. Associations between baseline scores of the major lipid subnetwork and the incidence of cardiovascular disease

	Quartiles of major subnetwork score[a]				HR per	P_{trend}
	Q1	Q2	Q3	Q4	1-SD increment	
Saturated phospholipid subnetwork						
Cases	55	75	49	51		
MV1[b]	Ref.	1.41 (0.93, 2.15)	1.10 (0.70, 1.73)	1.38 (0.86, 2.23)	1.12 (0.95, 1.32)	0.18
MV2[c]	Ref.	1.41 (0.92, 2.16)	1.11 (0.70, 1.76)	1.43 (0.87, 2.34)	1.14 (0.96, 1.35)	0.13
MV3[d]	Ref.	1.49 (0.96, 2.32)	1.37 (0.83, 2.23)	1.57 (0.94, 2.64)	1.20 (1.01, 1.44)	0.04
Unsaturated subnetwork						
Cases	72	65	47	46		
MV1	Ref.	0.97 (0.64, 1.46)	0.62 (0.40, 0.96)	0.63 (0.40, 1.00)	0.78 (0.65, 0.93)	0.006
MV2	Ref.	0.92 (0.61, 1.41)	0.61 (0.39, 0.96)	0.62 (0.39, 1.00)	0.78 (0.65, 0.93)	0.007
MV3	Ref.	1.00 (0.65, 1.55)	0.65 (0.42, 1.03)	0.71 (0.44, 1.15)	0.81 (0.67, 0.98)	0.03
Saturated glyceride subnetwork						
Cases	48	58	58	66		
MV1	Ref.	1.25 (0.80, 1.94)	1.28 (0.81, 2.02)	1.86 (1.18, 2.93)	1.28 (1.08, 1.52)	0.005
MV2	Ref.	1.29 (0.82, 2.04)	1.25 (0.79, 1.98)	1.81 (1.13, 2.90)	1.25 (1.04, 1.49)	0.02
MV3	Ref.	1.33 (0.82, 2.14)	1.18 (0.73, 1.92)	1.76 (1.09, 2.86)	1.22 (1.01, 1.47)	0.04
Monoglyceride subnetwork						
Cases	37	62	64	67		
MV1	Ref.	1.57 (0.99, 2.50)	1.73 (1.08, 2.76)	1.74 (1.09, 2.79)	1.18 (1.02, 1.38)	0.03
MV2	Ref.	1.57 (0.98, 2.51)	1.68 (1.04, 2.70)	1.74 (1.08, 2.78)	1.18 (1.01, 1.38)	0.04
MV3	Ref.	1.51 (0.93, 2.45)	1.59 (0.97, 2.61)	1.63 (0.99, 2.69)	1.15 (0.98, 1.36)	0.09

MV, multivariable model; ref., reference category.

[a]Quartiles were calculated based on the distributions of subnetwork score among the subcohort.

[b]Model 1 stratified on intervention group and simultaneously adjusted for age (continuous), sex (male, female) and other major subnetwork scores.

[c]Model 2 adjusted for body mass index (continuous), family history of cardiovascular disease (yes, no), and smoking status (current, never, former) in addition to model 1.

[d]Model 3 adjusted for histories of hypertension, dyslipidaemia and diabetes at baseline in addition to model 2.

Table 3, available as Supplementary data at *IJE* online). Every 1-SD increment in the score of the unsaturated subnetwork was associated with an HR of 0.73 (95% CI, 0.56-0.93) in the MedDiet group and an HR of 0.97 (95% CI, 0.71-1.32) in the control diet group.

Lipid clusters and CVD

We identified 10 lipid clusters and examined their associations with the incidence of CVD (Figure 2; and Supplementary Table 4, available as Supplementary data at *IJE* online). The ceramide cluster, the DAG and MAG cluster and the hydroxylated phosphatidylcholine (HPC) cluster included limited numbers of lipid metabolites and were each strongly associated with increased risk of CVD; the positive associations remained significant even after multiple comparison adjustment (Figure 2). The unsaturated phospholipid cluster predominantly consisted of phospholipids and CEs with more double bonds, and was associated with a decreased risk of CVD (HR per 1-SD increment in the cluster score = 0.83, 95% CI, 0.70-0.99, Figure 2). The saturated triglyceride cluster included a large number of DAGs and TAGs with saturated fatty acyl chains and was associated with an increased risk of CVD (HR per 1-SD increment in the cluster score = 1.20, 95% CI, 1.01-1.44, Figure 2). The score of the phosphocholine cluster, a cluster that comprised phospholipids with a smaller number of double bonds, was positively associated with CVD risk, but the association was marginally significant (HR per 1-SD increment in the pathway score = 1.17, 95% CI, 0.99-1.38, Figure 2). We observed many plasmalogen phospholipids in the plasmalogen cluster, and TAGs with a low degree of unsaturation in the saturated triglyceride cluster (Figure 2). The two lipid clusters were not associated with CVD risk in the final model.

Changes in lipid subnetworks/clusters

We observed similar trends from baseline to the 1-year follow-up in the lipid subnetwork/cluster scores when comparing each MedDiet group with the control group (Supplementary Table 6, available as Supplementary data at *IJE* online). Changes in the scores of lipid subnetwork/clusters from baseline to 1-year follow-up were generally

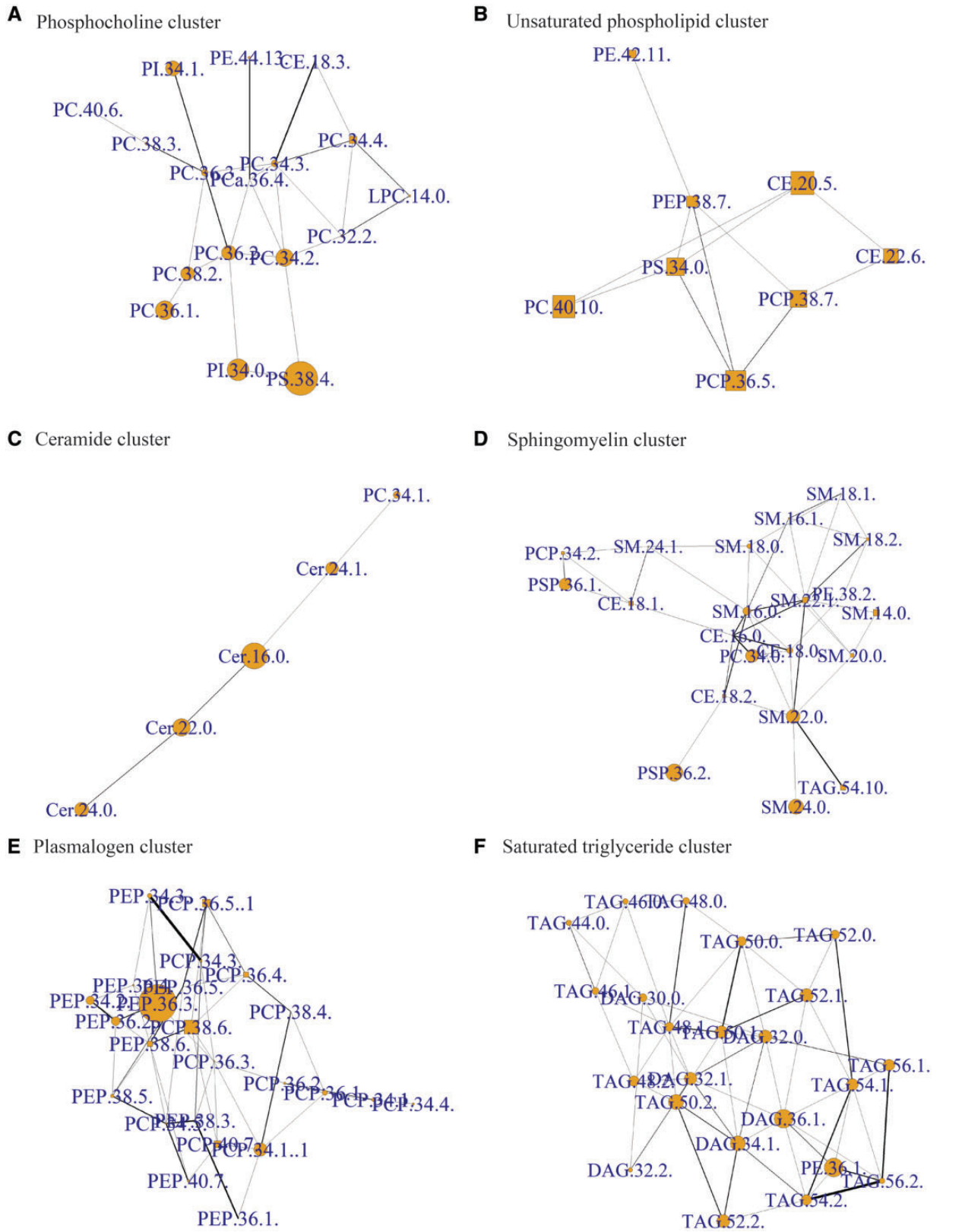

Figure 2. Lipid clusters and their associations with the incidence of cardiovascular disease in a subcohort of 787 participants and all 230 incident cases of cardiovascular diseases from the PREDIMED trial.

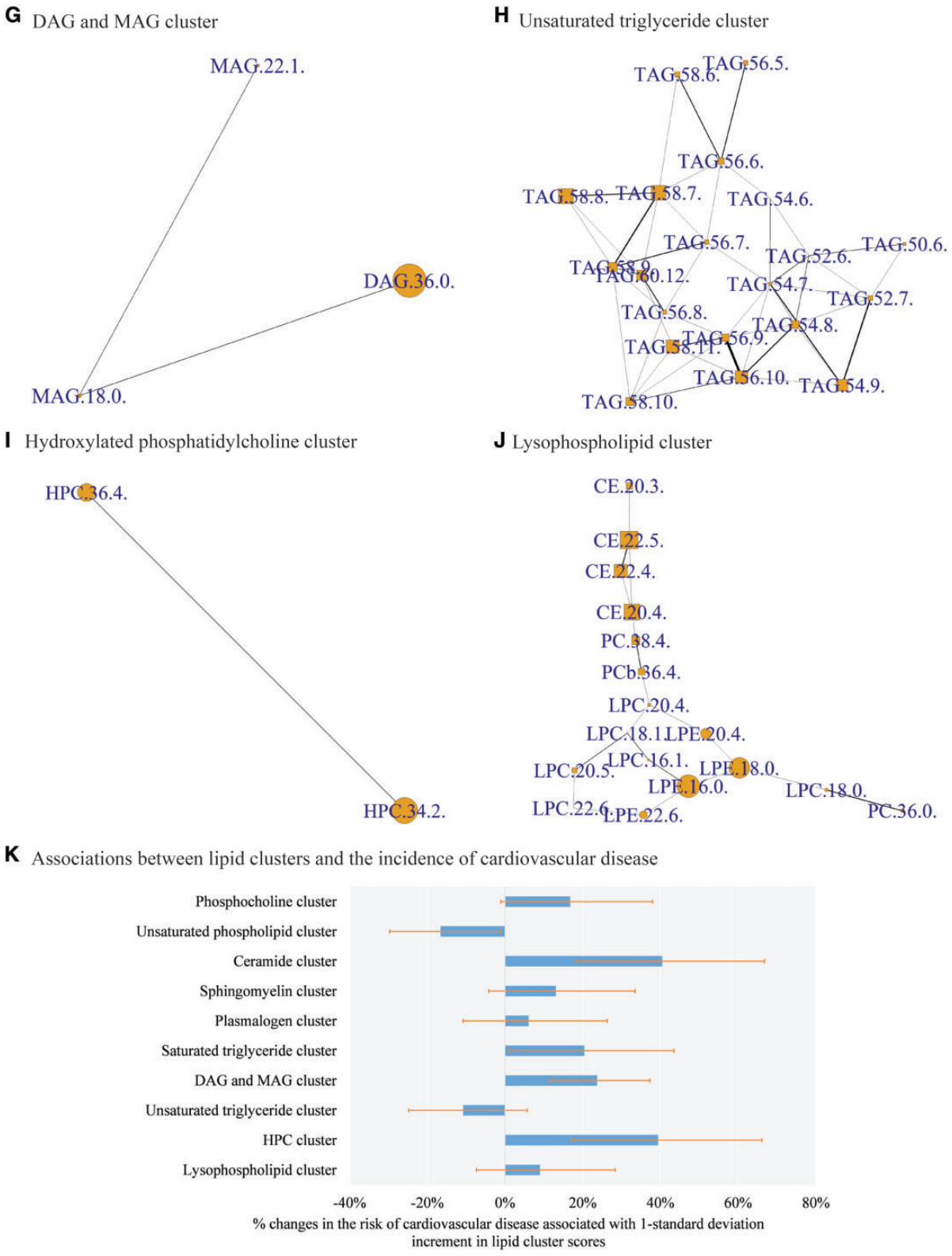

Figure 2. Continued

The small-scale lipid clusters represent pathways of lipid metabolism. The digits after the first period stand for number of carbon atoms on the fatty acyl chain. The digits after the second period stand for number of double bonds. Size of vertex is in proportion to −log (*P*-value) of the hazard ratio (HR) for the risk of composite cardiovascular disease endpoint associated with 1-standard deviation increment in the concentration of individual lipid metabolite. Square vertex corresponds to lipid metabolite associated with HR <1.00. Circle vertex corresponds to lipid metabolite with HR ≥ 1.00. Hazard ratios in Figure 2K were estimated from Cox regression model stratified by intervention group and simultaneously adjusted for age (continuous), sex (male, female), body mass index (continuous), family history of cardiovascular disease (yes, no), smoking status (current, never, former), histories of hypertension, dyslipidemia, and diabetes. Phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylethanolamine plasmalogen (PEP), phosphatidylserine (PS), phosphatidylserine plasmalogen (PSP), phosphatidylinositol (PI), Phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylcholine plasmalogen (PCP), hydroxylated phosphatidylcholine (HPC), cholesterol esters (CE), diacylglycerol (DAG), monoacylglycerol (MAG), triacylglycerol (TAG), sphingomyelins (SM), ceramide (Cer).

not associated with subsequent risk of CVD, except a suggestion of increased risk of CVD associated with the increase in score for the MAG subnetwork and MAG and DAG cluster (Supplementary Table 7, available as Supplementary data at *IJE* online).

Lipid subnetworks/clusters, conventional lipid markers and CVD

In the cross-sectional analysis on the lipid subnetwork/cluster scores and conventional lipid markers, participants with a higher score for the unsaturated subnetwork had significantly higher plasma levels of all four lipid markers (all P_{trend} <0.01; see Supplementary Table 8, available as Supplementary data at *IJE* online). The score for the saturated phospholipid subnetwork was positively associated with the levels of LDL-C, HDL-C and TC (all P_{trend} <0.001). We observed significantly positive associations between the score for the saturated glyceride subnetwork and levels of TG and TC, but a significantly inverse association between the score and level of HDL-C (all P_{trend} <0.001). The score for the MAG subnetwork was associated with none of the conventional lipid markers. Among the 10 lipid clusters, the scores for the phosphocholine cluster, unsaturated phospholipid cluster, plasmalogen cluster and lysophospholipid cluster were positively associated with the levels of all the lipid markers (all P_{trend} <0.05). Participants with a higher score for the sphingomyelin cluster had significantly higher levels of LDL-C, HDL-C and TC (all P_{trend} <0.001). The score for the ceramide cluster was associated with higher plasma levels of LDL-C, TC and TG (all P_{trend} <0.001), but was not associated with plasma HDL-C. We observed positive associations of the score for the saturated triglyceride cluster with the plasma levels of TC and TG, but an inverse association between the score and HDL-C (all P_{trend} <0.001). The score for the unsaturated triglyceride cluster was inversely associated with the level of HDL-C (P_{trend} = 0.008) but positively associated with the level of TG (P_{trend} <0.001). The DAG and MAG cluster and the HPC cluster were associated with none of the conventional lipid makers. Most associations between lipid subnetworks/clusters and CVD remained largely unchanged in the sensitivity analysis that adjusted for plasma levels of LDL-C, HDL-C and TG instead of baseline history of dyslipidaemia (Supplementary Table 5, available as Supplementary data at *IJE* online). Nevertheless, the associations for the saturated glyceride subnetwork and the saturated triglyceride cluster were attenuated, whereas the association for the unsaturated triglyceride cluster became stronger in the sensitivity analysis.

Discussion

We applied a network-based analytical approach to disentangle interactive relationships among lipid metabolites in the plasma lipidomics data from the PREDIMED trial. We detected four major subnetworks based on the topological structure of the global network, through which we found the degree of unsaturation of lipid metabolites as the major determinant of the general architecture of the global network. We also identified 10 biologically meaningful lipid clusters. By incorporating the structural information of lipid network into the framework of regression, we found divergent associations of the lipid subnetworks/clusters with CVD risk. Several lipid clusters, such as the HPC cluster and a cluster that included DAGs and a MAG with stearic acyl chain, strongly predicted CVD risk; these strong associations appeared to be independent of their degrees of unsaturation. There is a suggestion that the MedDiet intervention could modify the inverse association between the lipid subnetwork with high degree of unsaturation and CVD risk. However, the lipid subnetworks/clusters remained relatively stable under the MedDiet intervention during the first-year follow-up. Most lipid subnetworks and clusters showed significant associations with conventional lipid markers, whereas the DAG and MAG cluster and HPC cluster were not associated with the lipid markers. In addition, adjusting for levels of conventional lipids in the multivariable model did not materially attenuate the associations of most lipid subnetworks/clusters with CVD risk.

Largely driven by the degree of unsaturation of individual lipid metabolites, the initial clustering of the lipid metabolites generated four major subnetworks divergently associated with CVD risk. Our results corroborated Stegemann et al.'s findings that plasma lipid metabolites with more double bonds were generally associated with a lower risk of CVD, whereas those with fewer double bonds were generally associated with a higher risk of CVD in the Bruneck study.[4] Nevertheless, a recent lipidomics study within the ADVANCE (Action in Diabetes and Vascular Disease: PreterAx and DiamicroNMR Controlled Evaluation) trial failed to identify such a relationship between the degree of unsaturation of lipid metabolites, particularly for TAGs, and CVD risk.[6] However, the fact that all the participants in the ADVANCE study were type 2 diabetes patients might be one explanation for the difference from Stegemann et al.'s findings and our observations. Lipid metabolites with a high degree of unsaturation, including PCs, CEs, and TAGs that contain polyunsaturated fatty acids (PUFAs), particularly eicosapentanoic acid (EPA), docosahexanoic acid (DHA), docosapentaenoic acid (DPA), and arachidonic acid (AA), tended to cluster in

one subnetwork (unsaturated subnetwork). The high correlations among PUFA-rich lipid metabolites reflected that dietary intake is the predominant source of PUFAs contained in these metabolites. The observed association between this major subnetwork and decrease CVD risk was supported by the well-established evidence that high PUFA intake predicts a lower risk of CVD.[52–54]

We observed a stronger inverse association of the unsaturated subnetwork with CVD risk in the MedDiet intervention, compared to that in the control group, with a significant test of interaction. The MedDiet is characterized by the following: a high intake of virgin olive oil, fruit, nuts, vegetables, and cereals; a moderate intake of fish and poultry; a low intake of dairy products, red meat, processed meats, and sweets; and wine in moderation, consumed with meals.[55] In conjunction with two supplementary foods, extra-virgin olive oil or mixed nuts, the interventions in the PREDIMED trial might have improved dietary fat quality through decreasing saturated fat intake and increasing MUFA and PUFA intakes, and modulated *de novo* lipogenesis with higher intake of low-glycemic index food, and therefore boosted the cardio-protective effect of the subnetwork rich in PUFAs. Lipid metabolites with SFAs and MUFAs tended to clustered in three major subnetworks that were associated with higher CVD risk. Unlike PUFAs, the sources of SFAs and MUFAs contained in the lipid metabolites are diverse because they can be either derived from dietary sources or synthesized via the *de novo* lipogenesis pathway primarily in the liver. In addition, most dietary or endogenously synthesized SFAs are rapidly converted to MUFAs by the action of steroyl-CoA desaturase 1.[56] There is ample evidence supporting a mechanistic link of SFA and MUFA metabolism and high SFA intake, especially when substituted for PUFA, with CVD risk in part by promoting dyslipidemia.[57] Furthermore, atherogenic apolipoprotein B (ApoB)-containing lipoproteins preferentially carry MUFA-rich lipids; these ApoB-containing lipoprotein particles have enhanced ability to bind to arterial proteoglycans and retain in the artery wall.[58,59]

Among the small-scope lipid clusters, two were associated with lower risk of CVD. The unsaturated phospholipid cluster featured CEs and phospholipids that contain PUFAs and was inversely associated with CVD risk. The unsaturated triglyceride cluster was enriched by TAGs containing PUFAs and was only marginally associated with a lower risk of CVD. Mechanistic evidence supports that PUFA-rich CEs were causally linked to the development of atherosclerosis, but it is still debatable whether lipoprotein-associated TGs are mechanistically involved in the pathogenesis of CVD. Some argued that these TGs were simply carried together with CEs in the same lipoprotein class, rather than exerting causal functions in atherogenesis.[58,60]

It is possible that high abundance of PUFA-rich phospholipids is indicative of high anti-oxidative capacity because PUFAs are a primary target of oxidizing radicals during the phospholipid oxidation.[61] Oxidized phospholipids mediated the strong association between lipoprotein (a) and CVD risk through inducing monocyte trafficking to the arterial wall and pro-inflammatory responses.[62] Notably, we linked a lipid cluster (DAG and MAG cluster) featured by DAGs and a MAG that contained stearic acid to increased CVD risk. Previous mechanistic studies have showed that accumulation of DAGs, particularly those with SFA, led to the inhibition of insulin-stimulated insulin receptor kinase phosphorylation of the insulin receptor substrate proteins and an impaired activation of the downstream insulin-signaling cascade.[63–65] Meikle and colleagues reported decreased levels of circulating plasmalogens in patients with either stable or unstable coronary heart disease, compared with healthy controls, suggesting that a depletion of plasmalogens was indicative of oxidative stress and high risk of atherosclerosis.[66] However, our data did not support that the lipid cluster that included most plasmalogens (plasmalogen cluster) was associated with CVD risk. A possible explanation of the discrepancy could be that plasmalogen levels were indicative of plaque stability in Meikle et al's study, but not a predictor of atherogenesis and incident CVD in our study. Consistent with findings from previous studies, including our recent report from the PREDIMED trial,[67,68] the ceramide cluster was associated with elevated CVD risk. The role of ceramides in the development of insulin resistance has been intensively studied.[69–72] Human studies have observed positive correlations between plasma ceramide concentrations and inflammatory makers (e.g. interleukin-6[73] and TNF-α[74]) suggesting a relationship between excess ceramides and inflammation. Ceramides may contribute to plaque erosion[75] and cardiomyopathy.[76] The strong and positive association between the pathway that included two HPCs and CVD risk was novel and possibly suggested an important role of lipid oxidation in the pathogenic process of CVD.[77] A recent report from the Women's Health Initiative also found the positive associations of plasma HPCs with the risk of CVD.[78] Nevertheless, this finding should be further replicated in the future studies and investigated in mechanistic studies.

We explored the interrelationships among lipid subnetworks/clusters, conventional lipid markers, and CVD risk. The lipid subnetwork/cluster with a low degree of unsaturation (saturated glyceride subnetwork and saturated TAG cluster) was associated with high levels of TG and TC and low level of HDL-C, an atherogenic profile of conventional lipid markers. Moreover, the associations between the lipid subsets and the risk of CVD appeared to be mediated by mechanisms related to lipoprotein metabolism. On the

contrary, the lipid subsets (unsaturated subnetwork and unsaturated phospholipid cluster) that mainly consisted of lipid metabolites (particularly glycerides and phospholipids) with a high degree of unsaturation were associated with different lipid fractions in the same direction. Furthermore, the inverse associations between the lipid sets with a high degree of unsaturation and the risk of CVD remained largely unchanged or even became stronger after adjusting for conventional lipid markers in the model, suggesting a potential cardioprotective effect of the lipid sets beyond lipoprotein metabolism. These findings were important in the era when individuals can readily achieve recommended LDL-C levels through the use of lipid-lowering medication. With low LDL-C levels, the relative contribution of composition of lipoprotein particles other than CEs (e.g. TAGs and phospholipids) and the composition of fatty acid to CVD risk may increase.[79] The DAG and MAG cluster and HPC cluster were associated with none of the conventional lipid makers and were robustly associated with an increased risk of CVD independent of the conventional lipid markers, suggesting pathways (e.g. lipid oxidation and insulin-signaling pathways) other than lipoprotein metabolism that contributed importantly to the pathogenesis of CVD. All these observations further supported the added values of a detailed investigation of specific lipid metabolites compared with summary measures of lipids.

Our results should be interpreted in the context of several limitations. First, our lipidomics methods could not provide identification among isomers of lipid metabolite; molecular species that are more precise remain unknown. Secondly, participants were recruited based on their high CVD risk. Therefore, our findings might not be applicable in populations with low CVD risk. Thirdly, participants of this project were mostly European Caucasians, which might limit the generalizability of our findings to other populations. Fourthly, we cannot examine whether the results can be replicated in an independent population. Therefore, our findings should be interpreted as largely exploratory and warrant independent replication in the future. Finally, even though we carefully adjusted for many potential confounders, residual confounding could not be ruled out.

Our study possesses several major strengths. First, this study was built on a large, successful randomized controlled trial of primary prevention of hard clinical CVD endpoints with an intervention aiming to change the whole dietary pattern, which provided a unique and powerful setting to address our research questions, because of its well-characterized study population, high compliance to the interventions, and low rates of drop-out. Second, as a major advantage over traditional dimension-reduction methods (e.g. factor analysis and principle component analysis), our analytical approaches were able to identify many biologically meaningful and small-scope metabolic pathways and link these pathways to disease outcome, which naturally improved the interpretability of findings and facilitated the construction of follow-up hypotheses in a functional context. Third, the case-cohort design preserved the randomized design of this intervention trial and maintained the causal integrity of a randomized exposure status. Fourth, network-guided regression incorporated topological information as weights. This outcome-independent weighting scheme could address the concern over potential model overfitting embedded in using regression coefficients as weights to combine multiple metabolites (a popular weighting approach in metabolomics and lipidomics studies), and therefore improve robustness and reliability of statistical inference. Compared to unweighted subnetwork/cluster scores, this weighting scheme appeared to enhance the study efficiency for most subnetworks/clusters that have relatively large number of lipid metabolites (Supplementary Table 5, available as Supplementary data at *IJE* online).

In summary, by applying a network-based analytical method, our study constructed a global network of a large number of lipid metabolites and uncovered the lipid subnetworks and clusters that may indicate perturbed pathways involved in the pathogenesis of CVD. In addition, we directly incorporated topological information of network into a regression analysis and found that different metabolic pathways were divergently associated with CVD risk. Further studies are warranted to replicate these results in other populations and investigate potential mechanisms.

Supplementary Data

Supplementary data are available at *IJE* online.

Funding

This work was supported by a research grant from the National Institutes of Health [R01 HL118264]. The Prevención con Dieta Mediterránea (PREDIMED) trial was supported by the official funding agency for biomedical research of the Spanish government, the Instituto de Salud Carlos III, through grants provided to research networks specifically developed for the trial (RTIC G03/140 to R.E. and RTIC RD 06/0045 to Dr. Miguel A. Martínez-González) and through the Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición and by grants from: the Centro Nacional de Investigaciones Cardiovasculares (CNIC 06/2007); the Fondo de Investigación Sanitaria–Fondo Europeo de Desarrollo Regional (PI04–2239, PI 05/2584, CP06/00100, PI07/0240, PI07/1138, PI07/0954, PI 07/0473, PI10/01407, PI10/02658, PI11/01647, P11/02505 and PI13/00462); the Ministerio de Ciencia e Innovación (AGL-2009–13906-C02 and AGL2010–22319-C03); the Fundación Mapfre 2010, Consejería de

Salud de la Junta de Andalucía (PI0105/2007); the Public Health Division of the Department of Health of the Autonomous Government of Catalonia, Generalitat Valenciana (ACOMP06109, GVACOMP2010–181, GVACOMP2011–151, CS2010-AP-111, and CS2011-AP-042); and the Regional Government of Navarra (P27/2011). Dr. Dong D Wang was supported by a postdoctoral fellowship granted by the American Heart Association (16POST31100031).

Acknowledgements

We are very grateful to all the participants for their enthusiastic collaboration, the PREDIMED personnel for their excellent assistance and the personnel of all affiliated primary care centres.

Conflict of interest: None declared.

References

1. Quehenberger O, Dennis EA. The human plasma lipidome. *N Engl J Med* 2011;**365**:1812–23.
2. Fernandez C, Sandin M, Sampaio JL. Plasma lipid composition and risk of developing cardiovascular disease. *PLoS One* 2013;**8**:e71846.
3. Rhee EP, Gerszten RE. Metabolomics and cardiovascular biomarker discovery. *Clin Chem* 2012;**58**:139–47.
4. Stegemann C, Pechlaner R, Willeit P *et al*. Lipidomics profiling and risk of cardiovascular disease in the prospective population-based Bruneck study. *Circulation* 2014;**129**:1821–31.
5. Ganna A, Salihovic S, Sundström J *et al*. Large-scale metabolomic profiling identifies novel biomarkers for incident coronary heart disease. *PLoS Genet* 2014;**10**:e1004801.
6. Alshehry ZH, Mundra PA, Barlow CK *et al*. Plasma lipidomic profiles improve upon traditional risk factors for the prediction of cardiovascular events in type 2 diabetes. *Circulation* 2016;**134**:1637–50.
7. Chavez JA, Summers SA. A ceramide-centric view of insulin resistance. *Cell Metab* 2012;**15**:585–94.
8. Summers SA. Ceramides in insulin resistance and lipotoxicity. *Prog Lipid Res* 2006;**45**:42–72.
9. Cheng S, Shah SH, Corwin EJ *et al*. Potential impact and study considerations of metabolomics in cardiovascular health and disease: a scientific statement from the American Heart Association. *Circ Cardiovasc Genet* 2017;**10**:e000032.
10. Chuang HY, Lee E, Liu YT, Lee D, Ideker T. Network-based classification of breast cancer metastasis. *Mol Syst Biol* 2007;**3**:140.
11. Yousri NA, Mook-Kanamori DO, Selim MME-D *et al*. A systems view of type 2 diabetes-associated metabolic perturbations in saliva, blood and urine at different timescales of glycaemic control. *Diabetologia* 2015;**58**:1855–67.
12. Krumsiek J, Suhre K, Evans AM *et al*. Mining the unknown: a systems approach to metabolite identification combining genetic and metabolic information. *PLoS Genet* 2012;**8**:e1003005.
13. Krumsiek J, Suhre K, Illig T, Adamski J, Theis FJ. Gaussian graphical modeling reconstructs pathway reactions from high-throughput metabolomics data. *BMC Syst Biol* 2011;**5**:21.
14. Ried JS, Shin S-Y, Krumsiek J *et al*. Novel genetic associations with serum level metabolites identified by phenotype set enrichment analyses. *Hum Mol Genet* 2014;**23**:5847–57.
15. Shin S-Y, Fauman EB, Petersen A-K *et al*. An atlas of genetic influences on human blood metabolites. *Nat Genet* 2014;**46**:543.
16. Wang Z, Curry E, Montana G. Network-guided regression for detecting associations between DNA methylation and gene expression. *Bioinformatics* 2014;**30**:2693–701.
17. Wang Z, Maity A, Hsiao CK, Voora D, Kaddurah-Daouk R, Tzeng J-Y. Module-based association analysis for omics data with network structure. *PLoS One* 2015;**10**:e0122309.
18. Martinez-Gonzalez MA, Corella D, Salas-Salvado J *et al*. Cohort Profile: Design and methods of the PREDIMED study. *Int J Epidemiol* 2012;**41**:377–85.
19. Estruch R, Martinez-Gonzalez MA, Corella D *et al*. Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. *Ann Intern Med* 2006;**145**:1–11.
20. Estruch R, Ros E, Salas-Salvadó J *et al*. Primary prevention of cardiovascular disease with a mediterranean diet supplemented with extra-virgin olive oil or nuts. *N Engl J Med* 2018;**378**:e34.
21. U.S. Department of Health and Human Services and U.S. Department of Agriculture. *2015–2020 Dietary Guidelines for Americans*. 2015. http://health.gov/dietaryguidelines/2015/guidelines/ (8th, date last accessed).
22. Eckel RH, Jakicic JM, Ard JD *et al*. 2013 AHA/ACC guideline on lifestyle management to reduce cardiovascular risk: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *Circulation* 2014;**129**:S76–99.
23. Martínez-González MA, García-López M, Bes-Rastrollo M *et al*. Mediterranean diet and the incidence of cardiovascular disease: a Spanish cohort. *Nutr Metab Cardiovasc Dis* 2011;**21**:237–44.
24. Knoops KT, de Groot LC, Kromhout D *et al*. Mediterranean diet, lifestyle factors, and 10-year mortality in elderly European men and women: the HALE project. *JAMA* 2004;**292**:1433–39.
25. Fung TT, Rexrode KM, Mantzoros CS, Manson JE, Willett WC, Hu FB. Mediterranean diet and incidence of mortality from coronary heart disease and stroke in women. *Circulation* 2009;**119**:1093–100.
26. Sofi F, Cesari F, Abbate R, Gensini GF, Casini A. Adherence to Mediterranean diet and health status: meta-analysis. *BMJ* 2008;**337**:a1344.
27. Chrysohoou C, Panagiotakos DB, Pitsavos C, Das UN, Stefanadis C. Adherence to the Mediterranean diet attenuates inflammation and coagulation process in healthy adults: the ATTICA Study. *J Am Coll Cardiol* 2004;**44**:152–58.
28. Urpi-Sarda M, Casas R, Chiva-Blanch G *et al*. The mediterranean diet pattern and its main components are associated with lower plasma concentrations of tumor necrosis factor receptor 60 in patients at high risk for cardiovascular disease. *J Nutr* 2012;**142**:1019–25.
29. Esposito K, Marfella R, Ciotola M *et al*. Effect of a mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: a randomized trial. *JAMA* 2004;**292**:1440–46.
30. Mena MP, Sacanella E, Vazquez-Agell M *et al*. Inhibition of circulating immune cell activation: a molecular antiinflammatory effect of the Mediterranean diet. *Am J Clin Nutr* 2009;**89**:248–56.

31. Kastorini CM, Milionis HJ, Esposito K, Giugliano D, Goudevenos JA, Panagiotakos DB. The effect of Mediterranean diet on metabolic syndrome and its components: a meta-analysis of 50 studies and 534, 906 individuals. *J Am Coll Cardiol* 2011; **57**:1299–313.
32. Fito M, Guxens M, Corella D *et al*. Effect of a traditional Mediterranean diet on lipoprotein oxidation: a randomized controlled trial. *Arch Intern Med* 2007;**167**:1195–203.
33. Martinez-Gonzalez MA, Ruiz-Canela M, Hruby A, Liang L, Trichopoulou A, Hu FB. Intervention trials with the Mediterranean diet in cardiovascular prevention: understanding potential mechanisms through metabolomic profiling. *J Nutr* 2016;**146**: 913S–919S.
34. Ruano J, Lopez-Miranda J, Fuentes F *et al*. Phenolic content of virgin olive oil improves ischemic reactive hyperemia in hypercholesterolemic patients. *J Am Coll Cardiol* 2005;**46**:1864–68.
35. Fuentes F, Lopez-Miranda J, Perez-Martinez P *et al*. Chronic effects of a high-fat diet enriched with virgin olive oil and a low-fat diet enriched with α-linolenic acid on postprandial endothelial function in healthy men. *Br J Nutr* 2008;**100**:159–65.
36. Jones JL, Comperatore M, Barona J *et al*. A Mediterranean-style, low-glycemic-load diet decreases atherogenic lipoproteins and reduces lipoprotein (a) and oxidized low-density lipoprotein in women with metabolic syndrome. *Metabolism* 2012;**61**:366–72.
37. Langholz B, Thomas DC. Nested case-control and case-cohort methods of sampling from a cohort: a critical comparison. *Am J Epidemiol* 1990;**131**:169–76.
38. Prentice RL. A case-cohort design for epidemiologic cohort studies and disease prevention trials. *Biometrika* 1986;**73**:1–11.
39. Antman E, Bassand J-P, Klein W *et al*. Myocardial infarction redefined - a consensus document of the Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction. *J Am Coll Cardiol* 2000;**36**:959–69.
40. The European Stroke Organization (ESO) Executive Committee and the ESO Writing Committee. Guidelines for management of ischaemic stroke and transient ischaemic attack 2008. *Cerebrovasc Dis* 2008;**25**:457–507.
41. Morgenstern LB, Hemphill JC 3rd, Anderson C *et al*. Guidelines for the management of spontaneous intracerebral hemorrhage: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke* 2010;**41**: 2108–29.
42. Kidwell CS, Chalela JA, Saver JL *et al*. Comparison of MRI and CT for detection of acute intracerebral hemorrhage. *JAMA* 2004;**292**:1823–30.
43. Zipes DP, Camm AJ, Borggrefe M *et al*. ACC/AHA/ESC 2006 guidelines for management of patients with ventricular arrhythmias and the prevention of sudden cardiac death: a report of the American College of Cardiology/American Heart Association Task Force and the European Society of Cardiology Committee for Practice Guidelines (Writing Committee to Develop Guidelines for Management of Patients With Ventricular Arrhythmias and the Prevention of Sudden Cardiac Death). *J Am Coll Cardiol* 2006;**48**:e247–346.
44. Maradit-Kremers H, Nicola PJ, Crowson CS, Ballman KV, Gabriel SE. Cardiovascular death in rheumatoid arthritis: a population-based study. *Arthritis Rheum* 2005;**52**:722–32.
45. Schröder H, Fitó M, Estruch R *et al*. A short screener is valid for assessing Mediterranean diet adherence among older Spanish men and women. *J Nutr* 2011;**141**:1140–45.
46. Bluher M, Bashan N, Shai I *et al*. Activated Ask1-MKK4-p38MAPK/JNK stress signaling pathway in human omental fat tissue may link macrophage infiltration to whole-body insulin sensitivity. *J Clin Endocrinol Metab* 2009;**94**: 2507–15.
47. Clauset A, Newman ME, Moore C. Finding community structure in very large networks. *Phys Rev E* 2004;**70**:066111.
48. Kovsan J, Bluher M, Tarnovscki T *et al*. Altered autophagy in human adipose tissues in obesity. *J Clin Endocrinol Metab* 2011; **96**:E268–77.
49. Ravasz E, Somera AL, Mongru DA, Oltvai ZN, Barabási A-L. Hierarchical organization of modularity in metabolic networks. *Science* 2002;**297**:1551–55.
50. Barlow WE. Robust variance estimation for the case-cohort design. *Biometrics* 1994;**50**:1064–72.
51. Barlow WE, Ichikawa L, Rosner D, Izumi S. Analysis of case-cohort designs. *J Clin Epidemiol* 1999;**52**:1165–72.
52. Wang DD, Li Y, Chiuve SE *et al*. Association of specific dietary fats with total and cause-specific mortality. *JAMA Intern Med* 2016;**176**:1134–45.
53. Willett WC. Dietary fats and coronary heart disease. *J Intern Med* 2012;**272**:13–24.
54. Mozaffarian D, Wu JH. Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events. *J Am Coll Cardiol* 2011;**58**:2047–67.
55. Willett WC, Sacks F, Trichopoulou A *et al*. Mediterranean diet pyramid: a cultural model for healthy eating. *Am J Clin Nutr* 1995;**61**:1402–06S.
56. Fessler MB, Rudel LL, Brown M. Toll-like receptor signaling links dietary fatty acids to the metabolic syndrome. *Curr Opin Lipidol* 2009;**20**:379.
57. Mensink RP, Zock PL, Kester AD, Katan MB. Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. *Am J Clin Nutr* 2003;**77**: 1146–55.
58. Serfaty D, Rein M, Schwarzfuchs D *et al*. Abdominal fat subdepots and energy expenditure: magnetic resonance imaging study. *Clin Nutr* 2017;**36**:804–11.
59. Henkin Y, Kovsan J, Gepner Y, Shai I. Diets and morbid tissues - history counts, present counts. *Br J Nutr* 2015;**113**(**Suppl 2**): S11–18.
60. Shai I, Spence JD, Schwarzfuchs D *et al*. Dietary intervention to reverse carotid atherosclerosis. *Circulation* 2010;**121**:1200.
61. Krahmer N, Farese RV, Walther TC. Balancing the fat: lipid droplets and human disease. *EMBO Mol Med* 2013;**5**:973–83.
62. Wilfling F, Haas JT, Walther TC, Farese RV Jr. Lipid droplet biogenesis. *Curr Opin Cell Biol* 2014;**29**:39–45.
63. Thiam AR, Farese RV Jr, Walther TC. The biophysics and cell biology of lipid droplets. *Nat Rev Mol Cell Biol* 2013;**14**: 775–86.
64. Mayers JR, Wu C, Clish CB *et al*. Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. *Nat Med* 2014;**20**: 1193–98.

65. Wilfling F, Wang H, Haas JT *et al*. Triacylglycerol synthesis enzymes mediate lipid droplet growth by relocalizing from the ER to lipid droplets. *Dev Cell* 2013;**24**:384–99.
66. Meikle PJ, Wong G, Tsorotes D *et al*. Plasma lipidomic analysis of stable and unstable coronary artery disease. *Arterioscler Thromb Vasc Biol* 2011;**31**:2723–32.
67. Laaksonen R, Ekroos K, Sysi-Aho M *et al*. Plasma ceramides predict cardiovascular death in patients with stable coronary artery disease and acute coronary syndromes beyond LDL-cholesterol. *Eur Heart J* 2016;**37**:1967–76.
68. Wang DD, Toledo E, Hruby A *et al*. Plasma ceramides, Mediterranean diet, and incident cardiovascular disease in the PREDIMED trial. *Circulation* 2017;**135**:2028–40.
69. Adams JM, Pratipanawatr T, Berria R *et al*. Ceramide content is increased in skeletal muscle from obese insulin-resistant humans. *Diabetes* 2004;**53**:25–31.
70. Amati F, Dubé JJ, Alvarez-Carnero E *et al*. Skeletal muscle triglycerides, diacylglycerols, and ceramides in insulin resistance another paradox in endurance-trained athletes? *Diabetes* 2011;**60**:2588–97.
71. Dubé JJ, Amati F, Stefanovic-Racic M, Toledo FG, Sauers SE, Goodpaster BH. Exercise-induced alterations in intramyocellular lipids and insulin resistance: the athlete's paradox revisited. *Am J Physiol Endocrinol Metab* 2008;**294**:E882–88.
72. Straczkowski M, Kowalska I, Nikolajuk A *et al*. Relationship between insulin sensitivity and sphingomyelin signaling pathway in human skeletal muscle. *Diabetes* 2004;**53**:1215–21.
73. de Mello V, Lankinen M, Schwab U *et al*. Link between plasma ceramides, inflammation and insulin resistance: association with serum IL-6 concentration in patients with coronary heart disease. *Diabetologia* 2009;**52**:2612–15.
74. Haus JM, Kashyap SR, Kasumov T *et al*. Plasma ceramides are elevated in obese subjects with type 2 diabetes and correlate with the severity of insulin resistance. *Diabetes* 2009;**58**:337–43.
75. Holland WL, Summers SA. Sphingolipids, insulin resistance, and metabolic disease: new insights from in vivo manipulation of sphingolipid metabolism. *Endocr Rev* 2008;**29**:381–402.
76. Park T-S, Hu Y, Noh H-L *et al*. Ceramide is a cardiotoxin in lipotoxic cardiomyopathy. *J Lipid Res* 2008;**49**:2101–12.
77. Lee S, Birukov KG, Romanoski CE, Springstead JR, Lusis AJ, Berliner JA. Role of phospholipid oxidation products in atherosclerosis. *Circ Res* 2012;**111**:778–99.
78. Paynter NP, Balasubramanian R, Giulianini F *et al*. Metabolic predictors of incident coronary heart disease in women. *Circulation* 2018;**137**:841–53.
79. Mayr M, Gerszten R, Kiechl S. Cardiovascular risk beyond low-density lipoprotein cholesterol. *J Am Coll Cardiol* 2018;**71**:633–35.