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

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**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 ceramides7–8 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 studies2–6 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...
The present study applied a network-based analytical approach to lipomics 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 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.

**Study samples and lipomics profiling**

All analyses used fasting (fasting for ≥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 lipomics 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. 

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. 

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. 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. 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 the 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, 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. 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, 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., 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 associations of the lipid subnetworks/clusters with plasma lipids, 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 (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’ and ‘saturated glyceride subnetwork’ and ‘monoacylglyceride (MAG) subnetwork’, Figure 1 and Supplementary Figures-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 phosphatidylycholines (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 ($P_{\text{trend}} = 0.03$), but lower prevalence of diabetes and current smoking (both $p$-values $<0.001$) at baseline. The score for the unsaturated subnetwork was inversely associated with age ($P_{\text{trend}} <0.001$) and prevalence of diabetes ($P_{\text{trend}} = 0.02$), but positively associated with the prevalence of dyslipidaemia ($P_{\text{trend}} <0.001$). Participants with a higher score of the saturated glyceride subnetwork had higher BMI ($P_{\text{trend}} <0.001$) and prevalence of diabetes ($P_{\text{trend}} = 0.01$). 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, $P_{\text{trend}} = 0.007$) 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

<table>
<thead>
<tr>
<th>Intervention group</th>
<th>Cases</th>
<th>Subcohort</th>
<th>Quartiles of major subnetwork score&lt;sup&gt;d&lt;/sup&gt;</th>
<th>P-value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Saturated phospholipid subnetwork</th>
<th>Unsaturated subnetwork</th>
<th>Saturated glyceride subnetwork</th>
<th>Monoglyceride subnetwork</th>
<th>P-value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 230)</td>
<td>(n = 787)</td>
<td>Q1</td>
<td>Q4</td>
<td>Q1</td>
<td>Q4</td>
<td>Q1</td>
<td>Q4</td>
<td>Q1</td>
</tr>
<tr>
<td>Control</td>
<td>33 (36.1)</td>
<td>234 (29.7)</td>
<td>0.15</td>
<td>0.79</td>
<td>0.42</td>
<td>0.45</td>
<td>48 (24.5)</td>
<td>61 (31.0)</td>
<td>0.08</td>
</tr>
<tr>
<td>MedDiet + EVOO</td>
<td>35 (37.0)</td>
<td>291 (37.0)</td>
<td>0.48</td>
<td>0.32</td>
<td>0.79</td>
<td>0.79</td>
<td>79 (40.3)</td>
<td>77 (39.1)</td>
<td>0.31</td>
</tr>
<tr>
<td>MedDiet + nuts</td>
<td>20 (33.3)</td>
<td>68 (34.7)</td>
<td>0.32</td>
<td>0.32</td>
<td>0.79</td>
<td>0.79</td>
<td>69 (35.2)</td>
<td>59 (29.9)</td>
<td>0.31</td>
</tr>
<tr>
<td>Men, %</td>
<td>44 (19.1)</td>
<td>196 (24.9)</td>
<td>0.07</td>
<td>0.96</td>
<td>0.73</td>
<td>0.40</td>
<td>53 (27.0)</td>
<td>45 (22.8)</td>
<td>0.29</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>104 (45.2)</td>
<td>491 (62.4)</td>
<td>&lt;0.001</td>
<td>0.52</td>
<td>0.17</td>
<td>0.52</td>
<td>119 (60.7)</td>
<td>124 (62.9)</td>
<td>0.49</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>46 (20.0)</td>
<td>96 (12.2)</td>
<td>0.69</td>
<td>0.03</td>
<td>0.13</td>
<td>0.13</td>
<td>22 (11.2)</td>
<td>26 (13.2)</td>
<td>0.76</td>
</tr>
<tr>
<td>Dyslipidaemia, %</td>
<td>60 (34.8)</td>
<td>200 (25.4)</td>
<td>0.69</td>
<td>0.03</td>
<td>0.13</td>
<td>0.13</td>
<td>22 (11.2)</td>
<td>26 (13.2)</td>
<td>0.76</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>139 (60.4)</td>
<td>337 (42.8)</td>
<td>&lt;0.001</td>
<td>0.03</td>
<td>0.13</td>
<td>0.13</td>
<td>22 (11.2)</td>
<td>26 (13.2)</td>
<td>0.76</td>
</tr>
<tr>
<td>Age (years)</td>
<td>134 (58.3)</td>
<td>379 (47.3)</td>
<td>&lt;0.001</td>
<td>0.03</td>
<td>0.13</td>
<td>0.13</td>
<td>22 (11.2)</td>
<td>26 (13.2)</td>
<td>0.76</td>
</tr>
<tr>
<td>BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>29.6 ± 3.7</td>
<td>29.8 ± 3.6</td>
<td>0.63</td>
<td>0.03</td>
<td>0.13</td>
<td>0.13</td>
<td>22 (11.2)</td>
<td>26 (13.2)</td>
<td>0.76</td>
</tr>
<tr>
<td>Adherence to MedDiet</td>
<td>8.4 ± 1.8</td>
<td>8.8 ± 1.9</td>
<td>0.006</td>
<td>0.06</td>
<td>0.07</td>
<td>0.07</td>
<td>8.7 ± 1.9</td>
<td>9.0 ± 1.9</td>
<td>0.07</td>
</tr>
</tbody>
</table>

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

<sup>a</sup>The subcohort also included 37 cases.

<sup>b</sup>P<sup>2</sup> test was used for categorical variables and t test was used for continuous variables.

<sup>c</sup>To quantify a linear trend, we assigned the median within each quartile and modelled this score 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.

<sup>d</sup>Quartiles were calculated based on the distribution of the major subnetwork score in the subcohort.

<sup>e</sup>Adherence to the Mediterranean diet was assessed by a 14-item dietary screener.
CVD risk. The HRs of CVD, comparing extreme quartiles, were 1.76 (95% CI, 1.09-2.86, $P_{\text{trend}} = 0.04$) for the score of the saturated phospholipid subnetwork and 1.57 (95% CI, 0.94-2.64, $P_{\text{trend}} = 0.04$) 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 ($P_{\text{interaction}} = 0.04$, Supplementary 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
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 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

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

<table>
<thead>
<tr>
<th>Quartiles of major subnetwork score</th>
<th>HR per 1-SD increment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated phospholipid subnetwork</td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td></td>
</tr>
<tr>
<td>MV1a,b</td>
<td></td>
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<tr>
<td>MV2c</td>
<td></td>
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<tr>
<td>MV3d</td>
<td></td>
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<tr>
<td>Unsaturated subnetwork</td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td></td>
</tr>
<tr>
<td>MV1</td>
<td></td>
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<tr>
<td>MV2</td>
<td></td>
</tr>
<tr>
<td>MV3</td>
<td></td>
</tr>
<tr>
<td>Saturated glyceride subnetwork</td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td></td>
</tr>
<tr>
<td>MV1</td>
<td></td>
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<tr>
<td>MV2</td>
<td></td>
</tr>
<tr>
<td>MV3</td>
<td></td>
</tr>
<tr>
<td>Monoglyceride subnetwork</td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td></td>
</tr>
<tr>
<td>MV1</td>
<td></td>
</tr>
<tr>
<td>MV2</td>
<td></td>
</tr>
<tr>
<td>MV3</td>
<td></td>
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</tbody>
</table>

MV, multivariable model; ref., reference value.

aQuartiles were calculated based on the distributions of subnetwork score among the subcohort.
bModel 1 stratified on intervention group and simultaneously adjusted for age (continuous), sex (male, female) and other major subnetwork scores.
cModel 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.
dModel 3 adjusted for histories of hypertension, dyslipidaemia and diabetes at baseline in addition to model 2.
Figure 2. XXX.
Figure 2. Continued.
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.

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.6 Nevertheless, a recent lipidomics study within the ADVANCE (Action in Diabetes and Vascular Disease: PreterAx and DiamicronNMR 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 intake52–54 predicts a lower risk of CVD.

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 steryl-CoA desaturase. There is ample evidence supporting a mechanistic link of SFA and MUFA metabolism and high SFA intake, especially when substituted for PUFAs, with CVD risk in part by promoting hypercholesterolemia. 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. Among the small-scale 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 PUFAs-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. It is possible that high abundance of PUFAs-rich phospholipids is indicative of high anti-oxidative capacity because PUFAs are a primary target of oxidizing radicals during the phospholipid oxidation. Oxidized phospholipids mediated the strong association between lipoprotein (a) and CVD risk through inducing monocyte trafficking to the arterial wall and pro-inflammatory responses. 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 shown that accumulation of DAGs, particularly those with SFA, led to inhibition of insulin-stimulated insulin receptor kinase phosphorylation of the insulin receptor substrate proteins and an impaired activation of the downstream insulin-signaling cascade. 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. 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 PREMIDED trial, the ceramide cluster was associated with elevated CVD risk. The role of ceramides in the development of insulin resistance has been intensively studied. Human studies have observed positive correlations between plasma ceramide concentrations and inflammatory makers (e.g. interleukin-6 and TNF-α) suggesting a relationship between excess ceramides and inflammation. Ceramides may contribute to plaque erosion and cardiomyopathy. 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. A recent report from the Women’s Health Initiative also found the positive associations of plasma HPCs with the risk of CVD. 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 (particularlylycerides 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. 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 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/cluster 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 sub-networks 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.

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Conflict of interest: None declared.

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115. de Mello V, Lankinen M, Schwab U et al. Link between plasma ceramides, inflammation and insulin resistance: association with...


A Saturated phospholipid subnetwork (average unsaturation =0.07)

B Unsaturated subnetwork (average unsaturation =0.15)

C Saturated glyceride subnetwork (average unsaturation =0.06)

D Monoglyceride subnetwork 4 (average unsaturation =0.04)

Figure 1.
Figure 2.
**Figure 2.**

**G** DAG and MAG cluster

**H** Unsaturated triglyceride cluster

**I** Hydroxylated phosphatidylcholine cluster

**J** Lysophospholipid cluster

**K** Associations between lipid clusters and the incidence of cardiovascular disease

<table>
<thead>
<tr>
<th>Lipid Cluster</th>
<th>% Changes in the Risk of Cardiovascular Disease Associated with 1-standard Deviation Increment in Lipid Cluster Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphocholine cluster</td>
<td></td>
</tr>
<tr>
<td>Unsaturated phospholipid cluster</td>
<td></td>
</tr>
<tr>
<td>Ceramide cluster</td>
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<tr>
<td>Sphingomyelin cluster</td>
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<td>Plasmalogen cluster</td>
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<tr>
<td>Saturated triglyceride cluster</td>
<td></td>
</tr>
<tr>
<td>DAG and MAG cluster</td>
<td></td>
</tr>
<tr>
<td>Unsaturated triglyceride cluster</td>
<td></td>
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<tr>
<td>HPC cluster</td>
<td></td>
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<tr>
<td>Lysophospholipid cluster</td>
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</table>