Plasma lipidome patterns associated with cardiovascular risk in the PREDIMED trial: a case-cohort study

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Conflicts of interest

ER and RE received grants from the California Walnut Commission and personal fees from pharmaceutical and beverages companies. The remaining authors reported no conflicts of interest related to the study.

ABSTRACT

Background

The study of the plasma lipidome may help to better characterize molecular mechanisms underlying cardiovascular disease. The identification of new lipid biomarkers could provide future targets for prevention and innovative therapeutic approaches.

In the frame of the PREDIMED trial, our aim was to examine the associations of baseline lipidome patterns or their changes with the risk of clinical CVD events.

Methods

We included 983 participants in our case-cohort study. The end-point was the incidence of major CVD during 4.8 years of median follow-up. We repeatedly measured 202 plasma known lipid metabolites at baseline and after 1-year of intervention. Principal component analysis was used to identify lipidome factors. Among the 15 identified factors, 7 were significantly associated with CVD. Considering common patterns among factors, lipids were grouped (summed) into scores.

Results

After adjustment for traditional CVD risk factors, scores of baseline polyunsaturated phosphatidylcholines (PC)/lysoPC/PC-plasmalogens and polyunsaturated cholesterol esters (CE) showed inverse associations with CVD (p=0.032 and 0.009, respectively); whereas scores of monoacylglycerols (MAGs)/diacylglycerols (DAGs) and phosphatidylethanolamines (PEs) showed a direct association with CVD (p=0.024 and 0.016, respectively). Changes in PEs after 1-y of intervention were associated with higher CVD risk (p=0.033). We did not find a significant effect of the intervention with the Mediterranean Diet on these scores.

Conclusions

Our study suggests that polyunsaturated PCs and CEs may confer protection against CVD. In contrast, MAGs, DAGs and especially PEs appeared to be associated with higher CVD risk.

Keywords: Cardiovascular disease, lipidomics, Mediterranean diet, case-cohort, primary prevention.

1. INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of global mortality accounting for 17.9 million of deaths in 2015. In this scenario, prevention strategies to reduce the huge burden of CVD remain a high priority for public health. Dietary interventions are very likely to represent one of the most promising approaches for preventing CVD.

The PREDIMED study, a randomized trial for primary prevention of CVD with a nutritional intervention, revealed that the Mediterranean diet (MedDiet), supplemented with either extra virgin olive oil (EVOO) or nuts, reduced the risk of cardiovascular events by 30% after a median follow-up of 4.8 y [1].

Low levels of high-density lipoprotein cholesterol (HDL-C), high concentrations of low-density lipoprotein cholesterol and triacylglycerols (TAGs) are well established as independent predictors of CVD risk. However, some pharmacologic approaches aimed to improve lipid profiles have failed to reduce CVD risk [2]. Thus, there is a need to find further lipid biomarkers which may contribute to a better explanation and classification of CVD risk. The identification of these biomarkers may provide future targets for prevention and innovative therapeutic approaches.

Associations between some lipid metabolites and body mass index (BMI) [3], diabetes [4] and CVD [5] have been reported in studies of the lipidome. Hence, analyzing the lipidome in a randomized trial using a dietary intervention to modify the overall food pattern, would provide further insights in the search for new lipid biomarkers to better understand molecular mechanisms that may account for the benefits of a nutritional intervention. Moreover, the definition of the MedDiet *per se* and the results of PREDIMED have indicated that a relatively fat-rich dietary pattern may contribute to effective prevention of CVD. Thus, one of the potential mechanisms underlying this

beneficial effect of MedDiet may be explained by changes in the circulating lipidome. Beyond individual lipid species, it is interesting to identify combinations of molecules (i.e. lipid patterns) that may present a joint effect on the risk of CVD and may be changed by a dietary intervention with beneficial effects on hard CVD events. Therefore, we examined the associations of baseline lipidomic patterns or their 1-year changes with the risk of CVD in the PREDIMED trial.

2. MATERIALS AND METHODS

2.1. Study Population and Design

The PREDIMED study **(www.predimed.es)** is a randomized, primary cardiovascular prevention trial conducted in Spain in 7,447 participants at high vascular risk. The methods and design have been reported elsewhere [1,6] . Briefly, participants were randomly assigned to one of three nutritional interventions: MedDiet supplemented with 1) extra-virgin olive oil (MedDiet+EVOO), 2) or mixed nuts (MedDiet+Nuts), and 3) a control diet consisting of advice to reduce intake of fat.

For the present study, an unstratified case-cohort study nested in the PREDIMED trial was designed. We included a random sample of ~10% of PREDIMED participants at baseline and also 233 incident cases of CVD (55 of the 288 incident cases of the PREDIMED trial had no available plasma samples). We excluded 5 participants because of unavailable lipid metabolites data and 2 participants in the initial quality check. Finally, 983 participants were included in our analysis: 230 incident cases and 790 participants in the subcohort (including 37 overlapping cases). In addition, 907 participants (777 participants in the subcohort and 160 cases, including 30 overlapping cases) had available plasma samples after 1-year of follow-up and were included in the lipidome change analyses.

The Institutional Review Boards of all the recruitment centers approved the overall PREDIMED trial design according to the ethical guidelines of the Declaration of Helsinki. The Institutional Review Boards of the University of Navarra, the Broad Institute of MIT and Harvard, and the Harvard TH Chan School of Public Health, approved the case-cohort subproject. All participants gave written informed consent. Clinical trial registry number: Controlled-Trials.com number, ISRCTN35739639.

2.2. Lipidomics profiling

Fasting blood samples were collected at baseline and yearly thereafter during follow-up. Plasma EDTA tubes were aliquoted, coded and stored at −80 °C.

Pairs of samples (baseline and first-year) of the selected participants were randomly ordered and shipped on dry ice to the Broad Institute of Harvard&MIT for the metabolomics analyses. Specifically, plasma polar and nonpolar lipids were profiled. Detailed description about the method can be found in the supplemental material.

A total of 202 known lipid metabolites were analyzed for the present study.

2.3. Outcome Ascertainment

The primary endpoint of the PREDIMED trial was a composite outcome of non-fatal acute myocardial infarction, non-fatal stroke and cardiovascular death. Further details can be found in the supplemental material.

2.4. Covariate Assessment

At baseline and on yearly follow-up visits, a questionnaire was administered about lifestyle variables, educational achievement, history of illnesses, medication use, and family history of disease. Physical activity was assessed with the use of the validated Spanish version of the Minnesota Leisure-Time Physical Activity questionnaire [7]. A 137-item validated semi-quantitative food-frequency questionnaire was used by trained dietitians to ascertain dietary habits [8]. We used Spanish food-composition tables to

estimate energy and nutrient intakes [9]. Anthropometric and blood pressure measurements were directly measured and registered by trained nurses.

2.5. Statistical Analysis

Baseline characteristics of the participants, according to their primary outcome status, were described as means and standard deviations (SD) for quantitative traits and as percentages for qualitative traits.

Missing values for 30 lipid species (four of them with >5% of missing values and 26 with less than 1% of missing data) were replaced by the half of the minimum detected value, assuming that the missingness was a result of lower concentrations than the detectable threshold.

Baseline individual lipid values were normalized and scaled in multiples of 1 SD with Blom's inverse normal transformation [10]. Changes in the lipid values (1-y value minus the baseline value) were calculated and the resulting difference was also normalized and scaled.

The statistical assessment of the association between lipid patterns and CVD was done following two sequential steps: 1) We conducted an exploratory analysis using principal component analysis (PCA) to identify lipidome factors. PCA was performed considering the 202 lipid metabolites as candidates to be included in the obtained factors, and those factors with an eigenvalue higher than 2 were retained. Fifteen factors (not correlated) were extracted explaining 83% of the total variance. An orthogonal rotation (varimax) was used to better interpret the results. Individual metabolites with absolute loadings >0.40 were considered relevant components of the identified factors (table1S), as previously done based on convention [11]. Subsequently, Cox regression models weighted with Barlow weights [12] were used to calculate the hazard ratios (HR) and their 95% confidence interval (95% CI) for the risk of CVD for each factor

categorized into quartiles. Quartile cut-off points were generated considering only the subcohort and thereafter cases were categorized according to the same cut-off points. Each factor was introduced, as a continuous variable and categorized in quartiles, in an individual model adjusted for age, sex, BMI, smoking, family history of early coronary heart disease, leisure-time physical activity and intervention group and additionally adjusted for the rest of the PCA-identified factors. Similar models were used to evaluate the linear trend among factors considering the median value of each quartile as a quantitative variable. 2) After identifying the patterns of lipids commonly represented in those factors associated with CVD, we evaluated these lipid patterns grouping the metabolites based on their direct or inverse association with CVD and based on their lipid family trying to clarify biological mechanisms. Therefore, one score (lipid group A) resulted from the sum of all the metabolites represented among factors inversely associated with CVD and another one from the pattern of lipids identified within factors directly associated with CVD (lipid group B). Moreover, one individual score *per* each implicated lipid family was built by summing up the values of metabolites identified in the PCA, which belonged to the same lipid class (according to their chemical structure). Each lipid group (A and B) and each lipid family score were introduced as quartiles and as continuous in a weighted Cox regression model adjusted for age, sex, BMI, smoking habit, family history of early coronary heart disease, leisure time physical activity and stratified for intervention group to analyze their associations with incident CVD. Quartile cut-off points of each group or score were generated considering only the subcohort and thereafter cases were categorized according to the same cut-off points. Areas under the Receiver Operating Curves (AUC) were estimated to assess the predictive ability of each group or score beyond the already known predicting factors:

age, sex, BMI, smoking habit, family history of early coronary heart disease, leisure time physical activity and intervention group.

Our next step was to study the effects of changes in these lipid groups after 1-y intervention. Thus, following the same approach that at baseline, changes for each lipid group A and B, and one score for each implicated lipid family was calculated. After excluding those events occurred during the first year of intervention, each group or score of change was introduced (as continuous and quartiles) in a Cox regression model adjusted for its respective baseline score, and the same baseline confounding factors to analyze their effects on CVD risk.

All the statistical procedures were carried out with STATA 12.0 software. Statistical significance was set a priori at <0.05.

3. RESULTS

Baseline characteristics of the population according to their outcome status are shown in table 1. As expected, the subcohort presented similar baseline characteristics to those observed in the whole PREDIMED study [1]. We observed that cases presented higher levels of the classical cardiovascular risk factors than the subcohort members: older age and a higher proportion of men, smokers, diabetics and less active participants.

3.1. Factor analysis

Fifteen factors with eigenvalues≥2 were extracted from the PCA analysis conducted on 202 candidate lipid species measured at baseline. Seven extracted factors were significantly associated with CVD, 3 of them showed an inverse association with CVD and the other 4 were directly associated with CVD (Table 2). Supplementary table 1S shows the factor loadings for each factor. The association with a higher risk was especially strong for factor 11, mainly represented by phosphatidylethanolamines (PE).

Table 2S describe the lipid species included in each of these factors, which were found to be significantly associated with the risk of CVD.

3.2. Baseline lipid groups and CVD

Based on our first exploratory PCA results (table 3), in a second step we decided to group lipids according to whether the identified patterns were directly or inversely associated with CVD and also depending on their lipid families (chemical structures). We labeled lipid group A to the sum of that lipids, identified among factors, which were inversely associated with CVD and lipid group B to the sum of metabolites directly associated with CVD. Lipid group A was composed of three families of metabolites: 1) phosphatidylcholines (PC) family grouping PCs, LysoPCs and PC-plasmalogens presenting ≥5 double bonds (PC score); 2) cholesterol esters (CE) with >3 double bounds (CE score); and 3) long-chained triacylglycerols (TAG) with ≥52 carbon atoms containing ≥6 double bonds (long TAG score). For the lipid group B, four families of metabolites were identified: 1) all the monoacylglycerols (MAG) and diacylglycerols (DAG) (MAG & DAG score); 2) short-chained TAGs containing ≤4 double bonds (short TAG score); 3) PEs excluding those with saturated fatty acids (PE score) and 4) all the hydroxyPC (hPC) (hPC score). We also analyzed the association of each of the seven individual scores with CVD.

Baseline characteristics of the population and drug intake distribution according to the extreme quartiles of lipid groups A and B are presented in tables 3 and 4.

The results of the associations of both lipid groups A and B and each of the seven scores built according to lipid families with CVD are shown in table 5. For the lipid group A we found a statistically significant association with lower risk of CVD (p for linear trend=0.013). Within this lipid group, PC and CE scores were significantly associated with lower risk of CVD (p for linear trend 0.036 and 0.012, respectively). In the case of

the long TAGs score, we found a significant inverse association with CVD when considering it as a continuous variable (for each SD, HR: 0.84; 95% CI: 0.71-1.00). Lipid group B was significantly associated with a higher risk of CVD (p=0.006). When analyzing separately each family of lipids implicated in the group B, we observed that MAGs&DAGs, short TAGs and PEs scores were directly associated with a higher risk of CVD across their successive quartiles; however, the linear trend was only statistically significant for MAGs&DAGs and PEs scores (p=0.026 and 0.037, respectively). Nevertheless, we found a significant association for the 4 individual scores (MAG&DAG, short TAG, PE and hPC) when analyzing their effect as continuous variables (per each SD) (table 5).

When we additionally adjusted for the use of medication (statins, other lipid lowering drugs, insulin, other oral antidiabetics, antiplatelet agents, ACE inhibitors/AIIRA, diuretics, other antihypertensives and hormone replacement therapy), we found an attenuation of the effect (HR per each SD: 0.85; CI95%:0.69-1.04) in the association between baseline lipid group A and CVD. For lipid group B, we found no substantial differences in their association (HR per each SD: 1.29; CI95%:1.07-1.58) at baseline with CVD after adjustment for the use of medication. When the association between the 1-year changes in the lipids and CVD was considered, results hardly changed after adjustment for drugs (data not shown).

The predictive ability of both groups A and B was evaluated using ROC curve analyses, considering age, sex, BMI, smoking habit, family history of CVD, leisure time physical activity and intervention group as the basic model to assess the improvement in prediction by adding these groups of lipids. We observed that the lipid group B (Fig.1S) was able to significantly improve the prediction of CVD beyond that of risk factors

(AUC excluding lipid patterns= 0.692 (CI 95%:0.65-0.73) and AUC including lipid group B = 0.709 (CI 95%:0.67-0.75); p=0.0492 for the comparison).

3.3. Lipids groups changes and CVD

In order to analyze the potential effects of the intervention on lipidome changes, we considered the same baseline metabolite groups and, after calculating 1y-baseline changes, we calculated the scores of lipid changes.

We found that neither changes in the lipid group A nor changes in the group B were associated with CVD events occurring after 1 year (table 3S). When analyzing changes in each lipid family, we only found that the score reflecting 1-y changes in PEs presented a trend to be associated with an increased risk of CVD (p for linear trend=0.081) in consistency with our findings for baseline levels of PEs (tables 5 and 3S).

3.4. Effect of the predimed intervention on changes in lipidome factors

Finally, we analyzed the effects of the PREDIMED intervention on lipid changes by assessing the observed 1-y changes for each lipid group in each of the three arms of the trial. We did not observe any significant changes in these lipid groups for any of the 2 MedDiet groups in comparison with the control group (Fig.1). However, subjects allocated to MedDiet+EVOO presented a marginally significant trend to reductions in the lipid group B (associated with a higher risk of CVD at baseline) (Fig. 3b). This reduction was based on lower levels of short TAGs, MAGs, DAGs and PEs compared to the control group (Fig. 3b).

4. DISCUSSION

The present work was aimed to identify patterns of the lipidome that could account for reduced or increased CVD risk after a dietary intervention in the PREDIMED trial. Our main findings were: 1) at baseline, two different lipid patterns associated with CVD

were identified: a) one lipid pattern, composed by 3 families of metabolites, showed inverse association with CVD events (lipid group A): polyunsaturated PCs, lysoPCs, PC-plasmalogens, CEs, and long TAGs; b) the other pattern (lipid group B) was composed by 4 families of metabolites and showed a direct association with CVD: short TAGs (saturated/monounsaturated), hPCs and, especially, MAGs&DAGs and PEs. 2) Changes after 1-y of intervention pointed at the role of PEs on higher subsequent CVD risk. And 3) a 1-y intervention with MedDiet showed no sound effects on lipidome changes, with only trends to lower short TAGs, PEs, MAGs and DAGs in the group receiving intervention with MedDiet+EVOO.

A beneficial effect of dietary polyunsaturated fatty acids on CVD risk has been consistently reported in the literature [13,14]. However, studies related to plasma polyunsaturated status have yielded inconsistent results [15–17]. In our study, we observed that especially the PC lipid family and some CEs likely to be carrying eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) or long TAGs likely to be carrying EPA and arachidonic acid (AA) were associated with a lower CVD risk. This finding is in concordance with some results from other studies [18,19].

A higher risk of CVD in our population was mainly associated with high circulating levels of MAGs, DAGs, and PEs. Increased circulating levels of DAGs have been associated with visceral fat deposition [20] and in liver biopsies, short chain DAGs containing a small number of double bonds (≤3) have been associated with liver steatosis [21]. Moreover, in the Framingham study, a higher content of short TAGs with low number of double bonds was associated with insulin resistance [4]. In addition, in the Bruneck study, these lipids were associated with higher CVD risk [5]. These findings are highly consistent with our results, as we found an association of saturated/monounsaturated short TAGs and their precursors with higher CVD risk,

especially when taking into account that 64% of the CVD cases were diabetics and 45% were obese. Moreover, high levels of saturated and short DAGs in steatotic liver appeared to lead to altered levels of TAGs and PEs [21]. Other authors have found an imbalance of PCs/ PEs in the membrane of hepatocytes of fatty liver presenting an abnormally high content of PEs [22,23]. In fact, we found that PEs were the group of metabolites showing the strongest direct association with CVD risk. These findings may suggest that the risk of CVD explained by MAGs, DAGs, short saturated TAGs and PEs in our population may be linked to an excess of visceral fat, which disrupts the normal metabolism of abdominal tissues.

When we assessed changes in lipids after 1-y of intervention, we found that the association between increased levels of PEs and higher CVD risk after 1-y was persistent, supporting the hypothesis that PEs may play an important role in the development of CVD. Thus, it appears important to further study the mechanism of PEs in other tissues, beyond the liver, by which elevated levels of PEs may be one of the mechanisms underlying the development of future CVD events.

Finally, we observed no significant changes in metabolites due to the intervention with MedDiet. However, we observed a trend among subjects allocated to MedDiet+EVOO had reduced PEs, short TAGs, MAGs and DAGs after 1-year compared to the control group. This finding was consistent with the reduced CVD risk found for the MedDiet+EVOO group in PREDIMED study after a median follow-up period of 4.8 y [1]. Lack of statistical significance may be explained by the fact that 1 year represents a short induction period, and to the potential initial adaptation of several tissues responding to a progressive higher adherence to the MedDiet. Perhaps, our observation period for changes in the lipidome on 1y might be not long enough to observe the reflection in plasma of the stable cell metabolism of an established MedDiet pattern as

intended by our intervention. In addition, we discarded 126 lipid metabolites because they were not represented in the identified baseline factors (PCA), addressed to identify patterns associated with CVD, and we followed the same approach when looking at 1-y changes. Though, it is possible that MedDiet may be exerting its main effects trough other lipids, we did not find any robust evidence to support that substantial lipidome changes may account for the benefits observed in the PREDIMED trial. Other factors, such as changes in the antioxidant capacity [24] of the diet or enrichment of the anti-infammatory potential [25] of the diet might have been more important. In fact, some of the benefits of the MedDiet can be mediated by non-lipid mechanisms, such as the high intake of bioactive polyphenols with beneficial anti-oxidant and anti-inflammatory properties that are characteristic of the overall Mediterranean food pattern [26,27].

4.1. Study limitations

Some limitations of this study deserve to be acknowledged. It is possible, that with the initial PCA approach we have missed interconnections or interactions between lipid species/groups after 1-y of intervention that may better explain the potential mechanisms underlying changes caused by MedDiet. Second, our results may not be fully generalized to other populations because of the high baseline vascular risk presented in the sample of the PREDIMED trial. Third, the number of incident cases was relatively low and we may have suboptimal statistical power to detect some associations. And fourth, it would have been very interesting to have inflammation biomarkers data to test the hypothesis of the effects of both lipids and inflammation on CVD risk. Unfortunately, the data about inflammation biomarkers are limited to a very few participants among the case-cohort randomly selected for this substudy. These biomarkers were measured only to conduct the pilot study [28] in only a small subset (roughly 10%) of the PREDIMED participants.

4.2. Study strengths

Our manuscript presents important strengths. First, the design of the study enables us to both describe a baseline lipidome pattern labelling the initial risk of participants and 1-y changes driven by the intervention in a randomized controlled trial. Second, considering the whole lipidome we are capturing potential relationships between different lipid species and not only isolated effects of each lipid species.

5. CONCLUSIONS

In conclusion, our study suggests that specific patterns of lipid species carrying polyunsaturated fatty acids may confer protection against CVD, including PC, long-chain TAGs and CE. In contrast, PEs may play an important role in increasing CVD risk. In the same direction mono-, di- and short tri-acylglycerides, saturated or monounsaturated, appeared to contribute to CVD risk. Further assessments should explore, in further detail, the effect of interactions within lipids in networks or combined metabolic pathways to better understand the role of the dietary intervention in lipid-mediated changes in CVD risk.

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SUPPLEMENTAL MATERIAL

Lipidomics profiling method

Plasma polar and nonpolar lipids were profiled using a Nexera X2 U-HPLC system (Shimadzu Scientific Instruments; Marlborough, MA) coupled to an Exactive Plus orbitrap mass spectrometer (Thermo Fisher Scientific; Waltham, MA). Lipids were extracted from plasma (10 µL) using 190 µL of isopropanol containing 1,2-didodecanoyl-sn-glycero-3-phosphocholine as an internal standard (Avanti Polar Lipids; Alabaster, AL). After centrifugation (10 min, 9000×g, ambient temperature), supernatants (10 µL) were injected directly onto a 100 x 2.1 mm ACQUITY BEH C8 column (1.7 µm; Waters; Milford, MA). The column was eluted at a flow rate of 450 µL/min isocratically for 1 minute at 80% mobile phase A (95:5:0.1 vol/vol/vol 10 mM ammonium acetate/methanol/acetic acid), followed by a linear gradient to 80% mobile-phase B (99.9:0.1 vol/vol methanol/acetic acid) over 2 minutes, a linear gradient to 100% mobile phase B over 7 minutes, and then 3 minutes at 100% mobile-phase B. MS analyses were carried out using electrospray ionization in the positive ion mode using full scan analysis over m/z 200-1100 at 70,000 resolution and 3 Hz data acquisition rate. Additional MS settings were: ion spray voltage, 3.0 kV; capillary temperature, 300°C; probe heater temperature, 300 °C; sheath gas, 50; auxiliary gas, 15; and S-lens RF level 60. Raw data were processed using Progenesis QI software (NonLinear Dynamics) for feature alignment, nontargeted signal detection, and signal integration. Targeted processing of a subset of lipids was conducted using TraceFinder software (version 3.2, Thermo Fisher Scientific; Waltham, MA). Lipids are denoted by headgroup and total acyl carbon content and total acyl double bond content.

Outcome Ascertainment

Physicians, blinded with respect to the intervention, reviewed yearly, in each recruitment center, all the participants' medical charts to assess any incident CVD outcome. Other sources of information (also blinded with respect to the intervention), such as consultation of the National Death Index, were used to ascertain incident cases. Then, anonymized information was sent from each recruitment center to a blinded central Event Ascertainment Committee who finally adjudicated the events.

SUPPLEMENTAL AND ADDITIONAL RESULTS

Table 1S. Factor loadings for the seven extracted factors associated with CVD

Variable	Factor1	Factor2	Factor9	Factor10	Factor11	Factor12	Factor15
140lpc	0.70						
161lpc	0.41						
160lpc							
182lpc							
181lpc							
180lpc							
205lpc		0.43					
204lpc							
203lpc							
226lpc		0.41	0.45				
160lpe					0.43		
182lpe							
181lpe							
180lpe							
204lpe							
226lpe		0.44					
301pc	0.83						
300pc	0.82						
322pc	0.63						
321pc	0.75						
320pc	0.43						
344pc	0.59						
343pc	0.57			0.45			
342pc							
341pc	0.44						
340pc							
364pca							
364pcb							
363pc				0.52			
362pc							
361pc				0.41			
360pc							
386pc		0.62	0.60				
384pc							
383pc	0.49			0.61			
382pc				0.49			
4010pc		0.69					
409pc		0.64	0.61				
406pc	0.50			0.62			
345pcplasmalogen							

344pcplasmalogen					
343pcplasmalogen					
342pcplasmalogen					
341pcplasmalogena					
341pcplasmalogenb					
365pcplasmalogena		0.58			
365pcplasmalogenb					
364pcplasmalogen					
363pcplasmalogen					
362pcplasmalogen					
361pcplasmalogen					
387pcplasmalogen		0.46	0.53		
386pcplasmalogen					
384pcplasmalogen					
407pcplasmalogen					
320pe	0.73				
342pe	0.59				0.54
340pe	0.60				
364pe	0.51				0.64
363pe					
362pe	0.54				0.40
361pe	0.53				
360pe	0.44				
386pe		0.50			0.56
385pe					0.45
384pe					0.51
382pe					
406pe	0.46	0.50			0.44
343peplasmalogen					
342peplasmalogen					
365peplasmalogen					
364peplasmalogen					
363peplasmalogen					
362peplasmalogen					
361peplasmalogen					
387peplasmalogen		0.56			
386peplasmalogen					
385peplasmalogen					
383peplasmalogen					
407peplasmalogen					
4211peplasmalogen					
4413peplasmalogen					
341pi	0.41			0.50	
340pi					
364pi		0.54	0.62		
384pi					

340ps		0.72			
384ps					
386ps	0.72				
406ps	0.54				
363psplasmalogen					
362psplasmalogen					
361psplasmalogen					
sphingosine					
140sm					
161sm					
160sm					
182sm					
181sm					
180sm					
200sm					
221sm					
220sm					
241sm					
240sm					
160ceramided181					
220ceramided181					
240ceramided181					
241ceramided181					
140ce	0.63				
161ce	0.60				
160ce					
183ce	0.46			0.47	
182ce					
181ce					
180ce					
205ce		0.70			
204ce					
203ce				0.54	
226ce		0.47	0.64		
225ce					
224ce					
141mag					0.59
161mag					0.44
180mag					0.81
221mag					0.75
300dag	0.84				
322dag	0.75				
321dag	0.87				
320dag	0.87				
343dag	0.59				
342dag	0.68				

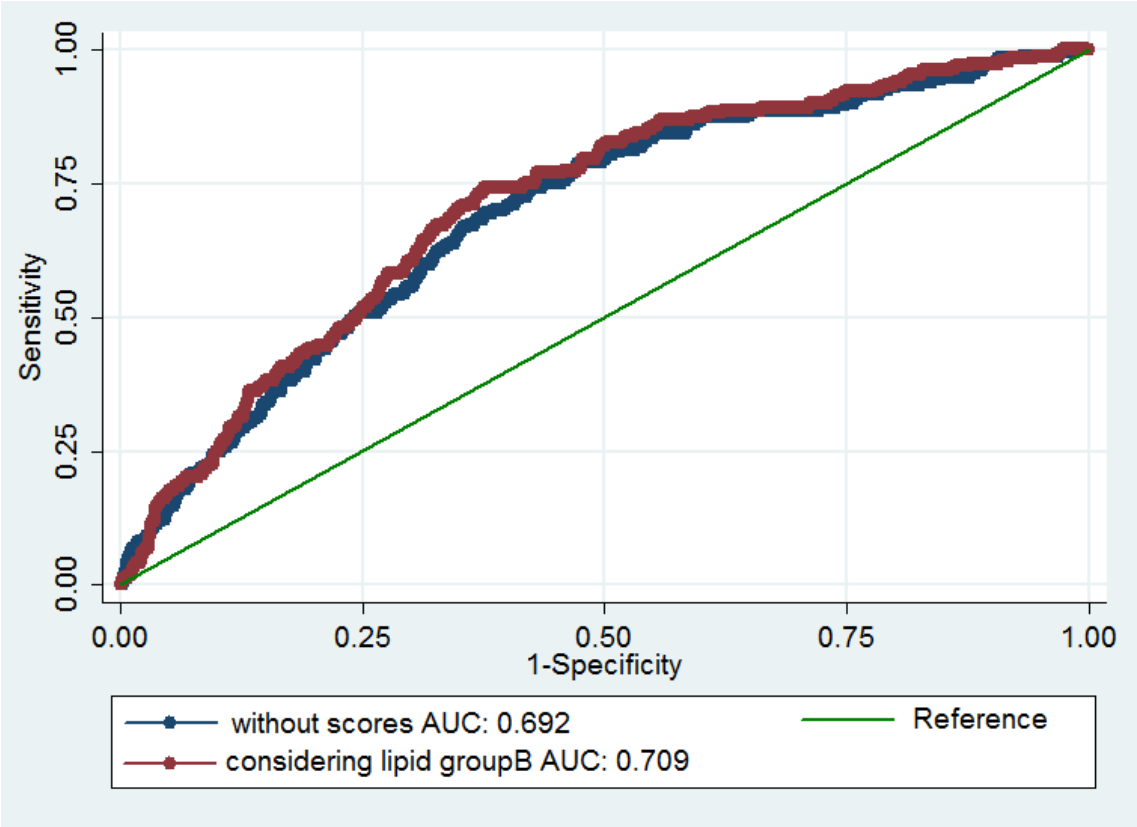
341dag	0.69		
340dag			
363dag			
362dag	0.41		
361dag	0.75		
360dag			0.62
364dag			
385dag	0.54		
384dag	0.50		
420tag	0.86		
442tag	0.92		
441tag	0.94		
440tag	0.93		
464tag	0.83		
463tag	0.90		
462tag	0.95		
461tag	0.96		
460tag	0.94		
485tag	0.79		
484tag	0.80		
483tag	0.87		
482tag	0.94		
481tag	0.93		
480tag	0.94		
506tag	0.73	0.52	
505tag	0.70		
504tag	0.65		
503tag	0.76		
502tag	0.83		
501tag	0.85		
500tag	0.93		
527tag	0.54	0.73	
526tag	0.53	0.58	
525tag			
524tag			
523tag			
522tag	0.55		
521tag	0.84		
520tag	0.86		
5410tag			
549tag		0.88	
548tag		0.84	
547tag		0.83	
546tag	0.41	0.66	
545tag			
544tag			

543tag				
542tag	0.48			
541tag	0.80			
5610tag		0.93		
569tag		0.93		
568tag		0.90		
567tag		0.86		
566tag		0.64		
565tag		0.40		
564tag				
563tag				
562tag	0.56			
561tag	0.74			
5811tag		0.96		
5810tag		0.93		
589tag		0.90		
588tag		0.81		
587tag		0.84		
586tag		0.63		
6012tag		0.94		
364ohpc				0.92
364ohpc				0.93
342ohpcma				0.92
342ohpc				0.91

Table 2S. Detailed description of factors extracted from PCA analysis and their association with CVD.

	Factor	Description	Main Components	Eigen value	Variance explained (%)
Inverse association with CVD	**2**	PhosphatidylCholines (PC), LysoPC (LPC), PC-plasmalogens carrying EPA and DHA Cholesterol Esters (CE) carrying EPA, DHA and DPA Long chain TAG (≥52C) carrying AA and DHA.	58:11TAG, 60:12TAG, 56:10TAG, 56:9TAG, 58:10TAG, 56:8TAG, 58:9TAG, 54:9TAG, 56:7TAG, 58:7TAG, 54:8TAG, 54:7TAG, 58:8TAG, 52:7TAG, 34:0PS, 20:5CE, 40:10PC, 54:6TAG, 40:9PC, 56:6TAG, 58:6TAG, 38:6PC, 36:5PCplasmalogen-a, 52:6TAG, 38:7PEplasmalogen, 36:4PI, 50:6TAG, 40:6PE, 38:6PE, 22:6CE, 38:7PCplasmalogen, 22:6LPE, 20:5LPC, 22:6LPC, 56:5TAG	29.5	14.6
	9		22:6CE, 36:4PI, 40:9PC, 38:6PC, 38:7PCplasmalogen,22:6LPC	4.6	2.3
	10		40:6PC, 38:3PC, 20:3CE, 36:3PC, 34:1PI, 38:2PC, 18:3CE, 34:3PC, 36:1PC	3.7	1.9
Direct association with CVD	**1**	MAG and DAG. Short chain TAG (<50 C) with 4 or less double bounds. Phosphatidylethanolamines (PE).	34:1PI, 54:6TAG, 36:2DAG, 16:1LPC, 32:0PC, 36:0PE, 34:1PC, 40:6PE, 18:3CE, 54:2TAG, 38:3PC, 40:6PC, 38:4DAG, 36:4PE, 36:1PE, 52:6TAG, 40:6PS, 52:7TAG 38:5DAG, 36:2PE, 52:2TAG, 56:2TAG, 34:3PC, 34:2PE, 34:3DAG, 34:4PC, 16:1CE, 34:0PE, 14:0CE, 32:2PC, 50:4TAG, 34:2DAG, 34:1DAG, 50:5TAG, 14:0LPC, 38:6PS, 50:6TAG, 32:0PE, 56:1TAG, 32:2DAG, 36:1DAG, 32:1PC, 50:3TAG, 48:5TAG, 48:4TAG, 54:1TAG, 30:0PC, 30:1PC, 50:2TAG, 46:4TAG, 30:0DAG, 52:1TAG, 50:1TAG, 52:0TAG, 42:0TAG, 32:0DAG, 32:1DAG, 48:3TAG, 46:3TAG, 44:2TAG, 44:0TAG, 50:0TAG, 48:1TAG, 48:0TAG, 46:0TAG, 48:2TAG 44:1TAG, 46:2TAG, 46:1TAG	49.9	24.7
	11	Hydroxy PC (adducts)	36:4PE, 38:6PE, 34:2PE, 38:4PE, 38:5PE, 40:6PE, 16:0LPE, 36:2PE	3.1	1.5
	12		36:4OHPC, 342OHPCMNA, 364OHPCMA, 342OHPC	2.9	1.5
	15		18:0MAG, 22:1MAG, 36:0DAG, 14:1MAG, 16:1MAG	2.2	1.1

Figure 1S. ROC curves for CVD risk excluding/including the direct pattern score and considering as predicting factors age, sex, BMI, smoking habit, family history of CVD, leisure time physical activity and intervention group.



AUC excluding lipid patterns= 0.692 (CI 95%:0.65-0.73)
AUC including lipid group B = 0.709 (CI 95%:0.67-0.75)
Chi^2 for the comparison of ROC curves = 5.59; **p=0.0492**

Table 3S. Association between scores of changes (1y-baseline) and CVD risk (adjusted for age, sex, BMI, smoking, family history of CVD and leisure-time physical activity).

	N of metabolites	Quartiles of scores				Linear trend	Per SD
		Q1	Q2	Q3	Q4		
1. PC [*] change score	10	Ref.	0.75 (0.43-1.30)	0.71 (0.40-1.25)	1.16 (0.68-1.98)	0.336	1.00 (0.80-1.25)
2. CE[†] change score	4	Ref.	1.00 (0.59-1.70)	0.81 (0.47-1.40)	1.04 (0.61-1.76)	0.939	0.96 (0.77-1.20)
3. Long TAG[‡] change score	14	Ref.	0.87 (0.50-1.52)	1.00 (0.58-1.80)	1.17 (0.67-2.05)	0.734	1.03 (0.83-1.28)
Lipid group A (1+2+3)	28	Ref.	0.73 (0.40-1.32)	1.05 (0.60-1.83)	1.11 (0.64-1.93)	0.390	1.01 (0.81-1.26)
4. MAG&DAG change score	19	Ref.	1.20 (0.69-2.09)	1.19 (0.67-2.09)	1.55 (0.90-2.65)	0.194	1.12 (0.94-1.35)
5. Short TAG[§] change score	15	Ref.	1.06 (0.60-1.89)	1.11 (0.62-2.01)	1.16 (0.69-1.96)	0.720	1.04 (0.85-1.25)
6. PE change score	10	Ref.	1.60 (0.92-2.78)	1.56 (0.90-2.72)	1.81 (1.02-3.20)	0.081	1.13 (0.92-1.39)
7. hPC change score	4	Ref.	0.90 (0.49-1.65)	0.85 (0.45-1.58)	0.93 (0.55-1.56)	0.927	1.04 (0.87-1.25)
Lipid group B change (4+5+6+7)	48	Ref.	1.02 (0.60-1.77)	1.27 (0.73-2.22)	1.34 (0.78-2.29)	0.356	1.11 (0.93-1.34)

[1] PC, LysoPC and PC-plasmalogens containing ≥5 double bonds, [2] CE containing ≥5 double bonds, [3] TAG with 54 or more carbon atoms and containing ≥5 double bonds, [4] TAG with a low number of double bounds (≤4) and <50C.

Table 1. Baseline characteristics of the study participants according to the outcome status.

Variables	Subcohort (n=790*)	CVD Cases (n=230)
Age	67.2 (5.9)	69.5 (6.5)
BMI	29.8 (3.6)	29.6 (3.7)
LTPA (METS-min/day)	258 (258)	237 (238)
Women (%)	57.1	39.6
Familiar history of CHD (%)	24.9	19.1
Type 2 diabetes (%)	47.1	64.8
Dyslipidemia (%)	73.5	58.3
Hypertension (%)	83.7	82.6
Smoking		
Non smokers (%)	62.3	45.2
Smokers (%)	12.3	20
Former smokers (%)	25.4	34.8
Total energy intake (Kcal/day)	2334 (615)	2365 (687)
Adherence to MedDiet†	8.82 (1.9)	8.43 (1.81)
Intervention group		
Control (%)	29.7	36.1
MedDiet+EVOO (%)	37.1	35.7
MedDiet+Nuts (%)	33.2	28.3

*Including 37 overlapping cases

†14-items questionnaire

LTPA: Leisure time physical activity

Table 2. Association between baseline lipid factors (PCA extracted) and CVD risk (adjusted for age, sex, BMI, smoking habit, family history of CVD, leisure time physical activity and intervention group)

	Quartiles of factors				Linear trend	Per SD
	Q1	Q2	Q3	Q4		
Factor 2*	Ref.	1.02 (0.59-1.74)	0.57 (0.32-1.03)	0.68 (0.38-1.22)	**0.010**	**0.75 (0.62-0.91)**
Factor 9*	Ref.	1.07 (0.63-1.84)	0.92 (0.53-1.60)	0.60 (0.33-1.08)	**0.028**	**0.80 (0.68-0.95)**
Factor10*	Ref.	0.76 (0.47-1.25)	**0.54 (0.31-0.93)**	**0.49 (0.28-0.87)**	**0.024**	**0.80 (0.68-0.95)**
Factor 1*	Ref.	0.94 (0.53-1.67)	**1.84 (1.03-3.30)**	1.67 (0.95-2.90)	**0.010**	1.17 (0.98-1.40)
Factor 11*	Ref.	**2.20 (1.20-4.03)**	**3.08 (1.70-5.58)**	**3.27 (1.75-6.09)**	**<0.001**	**1.42 (1.21-1.67)**
Factor 12*	Ref.	1.01 (0.58-1.78)	1.37 (0.77-2.42)	**1.48 (0.84-2.60)**	**0.032**	**1.41 (1.17-1.71)**
Factor 15*	Ref.	1.10 (0.60-2.04)	1.53 (0.84-2.79)	**1.81 (1.04-3.15)**	**0.032**	**1.20 (1.02-1.41)**

*Additionally adjusted for the rest of factors (1-15)

Table 3. Baseline characteristics and medication use among the study participants according to extreme quartiles of lipid group A.

	Lipid group A-Q1 (n=274)	Lipid group A-Q4 (n=244)
Age	68.8 (6.1)	66.7 (6.3)
BMI	29.7 (3.9)	29.5 (3.2)
LTPA (METS-min/day)	230 (220)	256 (244)
Women (%)	46	58
Familiar history of CHD (%)	22	25
Type 2 diabetes (%)	62	48
Dyslipidemia (%)	57	84
Hypertension (%)	81	84
Smoking		
Non smokers (%)	55	64
Smokers (%)	13	13
Former smokers (%)	32	23
Adherence to MedDiet†	8.57 (1.82)	8.93 (1.69)
Total energy intake (Kcal/day)	2390 (664)	2293 (630)
Intervention group		
Control (%)	30	35
MedDiet+EVOO (%)	35	38
MedDiet+Nuts (%)	35	27
Statins (%)	28	48
Lipid lowering drugs (%)	5	4
Insulin (%)	6	4
Other oral antidiabetics (%)	41	29
Antiplatelet agents (%)	28	20
ACE inhibitors/AIIRA (%)	55	48
Diuretics (%)	22	19
Other antihypertensives (%)	31	25
Hormone replacement therapy (%*)	2	2

*Calculated only for women

†14-items questionnaire

LTPA: Leisure time physical activity

Table 4. Baseline characteristics and medication use among the study participants according to extreme quartiles of lipid group B.

	Lipid group B-Q1 (n=242)	Lipid group B-Q4 (n=270)
Age	68.5 (5.8)	67.2 (6.4)
BMI	28.8 (3.8)	30.6 (3.7)
LTPA (METS-min/day)	271 (234)	246 (282)
Women (%)	48	57
Familiar history of CHD (%)	26	20
Type 2 diabetes (%)	44	59
Dyslipidemia (%)	66	74
Hypertension (%)	84	83
Smoking		
Non smokers (%)	55	62
Smokers (%)	17	14
Former smokers (%)	29	25
Adherence to MedDiet†	8.83 (1.79)	8.66 (1.81)
Total energy intake (Kcal/day)	2284 (611)	2333 (668)
Intervention group		
Control (%)	32	30
MedDiet+EVOO (%)	37	38
MedDiet+Nuts (%)	31	33
Statins (%)	37	34
Lipid lowering drugs (%)	2	7
Insulin (%)	5	6
Other oral antidiabetics (%)	28	38
Antiplatelet agents (%)	24	18
ACE inhibitors/AIIRA (%)	49	53
Diuretics (%)	24	23
Other antihypertensives (%)	25	29
Hormone replacement therapy (%*)	3	1

*Calculated only for women

†14-items questionnaire

LTPA: Leisure time physical activity

Table 5. Association between scores at baseline and CVD risk (adjusted for age, sex, BMI, smoking, family history of CVD, leisure-time physical activity and intervention group).

	N of metabolites	Quartiles of scores				Linear trend	Per SD
		Q1	Q2	Q3	Q4		
1. PC* score	10	Ref.	0.74 (0.49-1.12)	0.72 (0.47-1.11)	0.62 (0.38-1.02)	**0.036**	**0.78 (0.65-0.93)**
2. CE† score	4	Ref.	0.77 (0.51-1.16)	0.71 (0.45-1.10)	**0.59 (0.37-0.94)**	**0.012**	**0.71 (0.59-0.86)**
3. Long TAG‡ score	14	Ref.	0.75 (0.49-1.15)	**0.56 (0.34-0.90)**	0.93 (0.60-1.44)	0.472	**0.84 (0.71-1.00)**
Lipid group A (1+2+3)	28	Ref.	0.99 (0.66-1.49)	**0.52 (0.32-0.84)**	0.68 (0.43-1.07)	**0.015**	**0.76 (0.63-0.92)**
4. MAG&DAG score	19	Ref.	1.00 (0.62-1.61)	1.50 (0.96-2.34)	1.56 (0.97-2.50)	**0.026**	**1.31 (1.11-1.55)**
5. Short TAG§ score	15	Ref.	1.35 (0.86-2.13)	1.22 (0.76-1.96)	**1.72 (1.09-2.73)**	**0.037**	1.16 (0.98-1.38)
6. PE score	10	Ref.	0.86 (0.55-1.36)	1.05 (0.67-1.65)	**1.59 (1.02-2.49)**	0.066	**1.26 (1.05-1.51)**
7. hPC score	4	Ref.	0.68 (0.42-1.11)	0.98 (0.61-1.60)	1.24 (0.81-1.90)	0.105	**1.30 (1.07-1.57)**
Lipid group B (4+5+6+7)	48	Ref.	1.41 (0.89-2.23)	1.13 (0.70-1.83)	**2.10 (1.32-3.33)**	**0.006**	**1.32 (1.10-1.58)**

*PC, LysoPC and PC-plasmalogens containing ≥5 double bonds, †CE containing ≥5 double bonds, ‡ TAG with 54 or more carbon atoms and containing ≥5 double bonds, §TAG with a low number of double bounds (≤4) and <50C.