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Plasma lipidomic profiling and risk of type 2 diabetes in the PREDIMED trial.

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ABSTRACT

Objective Specific lipid molecular changes leading to type 2 diabetes (T2D) are largely unknown. We assessed lipidome factors associated with future occurrence of T2D in a population at high cardiovascular risk.

Methods This is a case-cohort study nested within the PREDIMED trial, with 250 incident T2D cases diagnosed during 3.8 years of median follow-up, and a random sample of 692 participants (639 non-cases and 53 overlapping cases), without T2D at baseline. We repeatedly measured 207 plasma known lipid metabolites at baseline and after 1-year of follow-up. We built combined factors of lipid species using principal component analysis, and assessed the association between these lipid factors (or their 1-year changes) and T2D incidence.

Results Baseline lysophosphatidylcholines and lysophosphatidylethanolamines (grouped as LP), phosphatidylcholine-plasmalogens (PC-PL), sphingomyelins (SM) and cholesterol esters (CE) were inversely associated with the risk of T2D (multivariable-adjusted p for linear trend=<0.001, <0.001, <0.001 and <0.001 respectively). On the contrary, baseline triacylglycerols (TAG), diacylglycerols (DAG) and phosphatidylethanolamines (PE) were directly associated with T2D (multivariable-adjusted p for linear trend<0.001, <0.001 and <0.001, respectively). One-year lipid changes showed associations in similar directions (though non-significant after adjustment for baseline levels). TAG with odd-chain fatty acids showed inverse associations with T2D once adjusted for total TAG.

Conclusions

Two plasma lipid profiles, grouping different lipid classes, were found associated with T2D in participants at high cardiovascular risk: one profile including LP, PC-PL, SM

and CE associated with a reduced risk of T2D and another profile composed of TAG, DAG and PE associated with higher risk.

INTRODUCTION

Type 2 diabetes (T2D) is a metabolic disorder which, in most of the cases, occurs later in life, resulting from insulin resistance and an inadequate compensatory insulin secretion (1,2). In 2015, 415 million adults (8.8%) worldwide suffered from T2D, and it is estimated that this prevalence will increase to 642 million (10.4%) in 2040 (3). These data raised the importance for public health of preventing this disease and tackling the epidemic.

The etiopathogenesis of T2D is not fully understood but the major risk factors are well established: obesity, sedentary lifestyles, hypertension and dyslipidemia characterized by high plasma triglyceride concentration, low HDL cholesterol concentration and increased concentration of small dense LDL-cholesterol particles (4,5). However, under the umbrella of dyslipidemia, triglycerides consist of a large number of individual molecular species while lipoproteins are constituted by many different lipid classes containing multiple molecular species (6). The role that individual molecular species of lipids play on T2D development remains unclear. Lipidomics may contribute to the understanding of the biological mechanisms underlying the link between dyslipidemia and T2D. Moreover, it is known that hypercaloric and low-quality diets lead to an excess of fat depositions in the body, which is enhanced by insulin resistance leading to a status of lipototoxicity associated with T2D (7).

Thus, the PREDIMED study, an intervention trial with Mediterranean diet (MedDiet), seems to be an adequate scenario to both find lipidome profiles associated with T2D incidence and to discern if the intervention on diet lead to changes on the lipidome determining the risk of T2D.

Our aims were to: 1) assess lipidome patterns associated with subsequent risk of incident T2D, and 2) analyze if 1 y-changes in these lipid patterns induced by the intervention on diet were associated with subsequent T2D risk.

MATERIAL AND METHODS

The present study was designed as an unstratified case-cohort study nested in the PREDIMED trial (www.predimed.es), a Spanish primary cardiovascular prevention trial using Mediterranean diet as the main intervention. The methods and design of PREDIMED were previously described elsewhere (8,9). Briefly, 7,447 participants (men 55-80 and women 60-80 years old) who were initially free of cardiovascular disease (CVD) but at high cardiovascular risk were allocated to 3 dietary interventions: 1) a Mediterranean diet supplemented with extra-virgin olive oil (MedDiet+EVOO); 2) a Mediterranean diet supplemented with mixed nuts (MedDiet+nuts); or 3) a control diet consisting of advice to reduce the intake of all types of fat. Inclusion criteria were to have either T2D or 3 or more major cardiovascular risk factors. In the full PREDIMED cohort, 3,541 participants did not have T2D at baseline. Among the latter, we observed 273 incident cases of T2D during the trial follow-up. Participants who were randomized to the MedDiet+EVOO (or both MedDiets combined) had a significantly lower risk of T2D incidence compared with the control group (25).

The present case-cohort study comprises a random selection of 694 non-diabetic participants (approximately 20%) from the eligible subjects of the PREDIMED cohort who were free of diabetes at baseline and had available EDTA plasma samples, together with all incident cases of T2D occurring during a median follow-up of 3.8 years of intervention with available samples (samples were unavailable for 22 out of the 273 incident T2D cases occurring in the PREDIMED trial). Lipid metabolites were

measured for 889 participants included in the case-cohort, 639 were in the subcohort (including 53 overlapping cases) and 197 were the rest of the T2D cases, yielding a total of 250 incident cases (Figure 1S). In addition, 658 participants (501 non-cases and 157 cases occurred after 1-y follow-up) had 1-year follow-up samples and were included in the 1-year change analyses (Figure 1S).

The Institutional Review Boards of the recruitment centers approved the study protocol, and participants provided written informed consent.

Covariate assessment

At baseline and at yearly follow-up visits, a questionnaire about lifestyle variables, educational achievement, history of illnesses, medication use, and family history of disease was administered. Physical activity was assessed using the validated Spanish version of the Minnesota Leisure-Time Physical Activity questionnaire (26). Participants were considered to have dyslipidemia or hypertension if they had previously been diagnosed, and/or they were being treated with cholesterol-lowering, or antihypertensive agents, respectively. Trained personnel recorded anthropometric and blood pressure measurements.

Study samples and metabolite profiling

Fasting blood samples were collected at baseline and after 1-year of follow-up. After an overnight fast, plasma EDTA tubes were collected and aliquots were coded and kept refrigerated until they were stored at -80°. In June 2015, pairs of samples (baseline and first-year visits from each participant) were randomly ordered and shipped on dry ice to the Broad Institute of Harvard & MIT for the metabolomics analyses. Specifically, 207 plasma polar and nonpolar lipids were profiled. Detailed description about the analytical methods used can be found in previous publications by our group (10,11) and the supplemental material.

Clinical assessment

The PREDIMED protocol included T2D as a pre-specified secondary endpoint. The adjudication of new diagnoses of T2D during follow-up was conducted by the Clinical Endpoint Committee of PREDIMED, an ad hoc panel of medical doctors, as it has been described elsewhere (9,12). The criteria of the American Diabetes Association (1), namely two confirmations of fasting plasma glucose ≥7.0 mmol/L or 2-h plasma glucose ≥11.1 mmol/L, after a 75-g oral glucose load were used to adjudicate cases. The Clinical Endpoint Committee was blinded to the intervention group and to the identity of the patients.

Statistical analysis

Missing values for 26 lipid metabolites (four of them with >5% of missing values and 22 with less than 1% of missing data) were replaced by the half of the minimum detectable value, assuming that 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 (13). Changes in 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 T2D was conducted by 3 sequential steps.

Factor analysis: lipid *factors*

The first step was an exploratory principal component analysis (PCA). PCA was performed considering the 207 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 84% 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 (14). To analyze the association of each extracted factor with T2D, Cox regression models weighted with Barlow weights (15) were fitted. Each factor was introduced in the model either as a continuous variable or categorized in quartiles and adjusted for age, sex, intervention group and for the rest of the PCA-identified factors. Quartile cut-off points were generated considering only the subcohort and thereafter cases were categorized according to the same cut-off points.

Similar models were used to evaluate the linear trend among factors considering the median value of each quartile as a quantitative variable.

Grouping by lipid families: lipid *scores*

After identifying PCA-factors associated with T2D risk, our second step was to evaluate the association between the main lipids represented in those PCA-factors and T2D risk. In this second step, lipid molecular species were grouped (summed) into individual scores based on their lipid class (according to their chemical structure), thus trying to clarify potential biological mechanisms. The difference between lipid *factors* (obtained only through the data-driven PCA) and lipid *scores* is that in lipid *scores* both the known chemical structure and the data-driven result obtained with PCA were taken into account.

Weighted Cox regression models were applied to estimate hazard ratios (HRs) of T2D for participants in quartiles 2-4 versus the lowest quartile of each lipid *score*. We used the weighting scheme suggested by Barlow (15) to account for the overrepresentation of cases. To quantify a linear trend, we assigned the median value of each score within each quartile and modeled this variable continuously. We also estimated HRs of T2D

associated with a 1-standard deviation (SD) increment in the transformed lipid scores. Three parallel Cox regression models were designed according to their adjustment: 1)Model 1(M1): age, sex and intervention group, 2) Model 2 (M2): M1 additionally adjusted for BMI, smoking, leisure-time physical activity, hypertension and dyslipidemia and 3) Model 3 (M3): M2 additionally adjusted for baseline glucose (continuous+quadratic term).

To more deeply analyze the effects of each lipid score, HRs for incident T2D per 1 SD in baseline individual lipid concentrations with weighted Cox regression models using the M2 adjustment (see above) were calculated. The HRs for individual lipids and their p values were plotted, according to the previously defined lipid scores, in a 2-dimensional graph defined by the number of carbon atoms (x axis) and the number of double bonds (y axis) in the acyl chain, as we previously reported for cardiovascular disease (11). Lipids with the same number of carbon atoms and double bonds were slightly pulled apart horizontally to visualize both results. We also included an additional graph to plot the residual of each triacylglycerol over the total content of the considered triacylglycerol, due to the fact that hypertriglyceridemia is an already known risk factor for T2D (16).

Areas under the receiver operating curves (AUC) were estimated to assess the discrimination of each score beyond the already known predicting factors of T2D: age, sex, BMI, smoking, hypertension, dyslipidemia, leisure-time physical activity, intervention group and baseline glucose concentrations.

<u>One-year changes in lipid scores</u>

Our third step was to study the effects of changes in these lipid scores after 1-y intervention. Changes for each lipid score were used as the main exposure variable. After excluding T2D cases occurred during the first year of intervention, each score of

change was introduced (as a continuous variable or in quartiles) in Cox models adjusted for its respective baseline score, and reproducing the same models used to analyze their effects on T2D risk.

Finally, we modeled the risk of T2D per 1 SD in the 1-y change in individual lipid concentrations with the use of weighted Cox regression models using the M2 adjustment (described above) plus an additional adjustment for baseline lipid concentration. Again, HRs and their p values were grouped according to lipid scores and plotted in a 2-dimensional graph defined by the number of carbon atoms (x axis) and the number of double bonds (y axis) in the acyl chain.

Statistical significance was set a priori at <0.05.

RESULTS

Baseline characteristics of the population according to their diabetic incident status are shown in table 1. We observed that subjects who developed T2D during follow-up showed a mean fasting glucose concentrations of 117±18 mg/dl at baseline, suggesting that most of them might be already in a pre-diabetic status. T2D cases were more likely to be men and smokers.

Factor analysis

Fifteen factors with eigenvalues≥2 were extracted from the PCA analysis conducted on 207 candidate lipid metabolites measured at baseline (table 1S). Seven of them presented a statistically significant association with T2D incidence: four of them were directly associated and the other 3 inversely associated with T2D incidence (table 2). Table 2S describes each factor and its components. Lysophospholipids (LP), cholesterol esters (CEs), sphingomyelins (SM) and phosphatidylcholines plasmalogens (PC-PLs) were widely represented among factors associated with lower T2D risk. On the other

hand, triacylglycerols (TAGs), diacylglycerols (DAGs) and phosphatidylethanolamines (PEs) were preponderantly associated with higher risk.

Baseline lipid scores

Based on the lipid patterns observed among the extracted factors in PCA (tables 2 and 2S), seven classes or families of lipids (according to their common chemical structures) were identified. The identified metabolites belonging to each lipid class were summed up to build the following scores: 1) LP score, grouping lysophosphatidylcholines (LPC) and lysophosphatidylethanolamines (LPE) (n of metabolites=18); 2) PC-PL score (n of metabolites =15); 3) SM score (n of metabolites=11); 4) CE score (n of metabolites=13); 5) TAG score, including only those TAG with ≤56C and ≤3 double bonds (n of metabolites =40); 6) DAG score (n of metabolites=14), and 7) PE score (n of metabolites=12).

Table 3 shows the association of each score with T2D. Higher LP, PC-PL, SM, and CE baseline scores presented a statistically significant inverse association with T2D (p for linear trend=<0.001, <0.001, <0.001 and <0.001, respectively; adjusted for sex, age and intervention group). These associations were maintained after additional adjustment for BMI, smoking, leisure time physical activity, hypertension and dyslipidemia (table 3). When fasting baseline glucose levels were introduced in the models, the inverse association was maintained for PC-PL, SM and CE scores (p for linear trend=0.004, 0.001 and 0.016, respectively) and was attenuated for LP score (p for linear trend=0.053).

On the contrary, higher TAG, DAG and PE baselines scores presented a direct association with T2D (table 3) both after adjustment for sex, age and intervention group-adjustment (p for linear trend=<0.001, <0.001 and <0.001, respectively) and in the fully-adjusted model (p for linear trend= <0.001, <0.001 and 0.002 respectively).

Further adjustments for baseline glucose showed that the association between the DAG score and T2D was robust (p for linear trend=0.017). The association between TAGs and incident T2D was attenuated but it remained still evident (table 3).

In figure 1, we show associations for each individual lipid within each family represented according to number of carbon atoms and to its number of double bonds in the acyl chain. In figure 1a, we observed that LP, SM and CE were the most homogenous lipid groups regarding their individual associations with T2D (inverse associations). Moreover, we did not find among the scores any clear pattern of inverse association with T2D incidence depending on the number of C atoms or double bounds. As shown in figure 1b, a clear direct association of TAG, DAG and PE scores with T2D was evident. We observed no direct association of the number of C atoms or double bounds with the risk of T2D for the DAG and PE groups. However, for TAGs we observed a strong attenuation of the direct association with T2D for odd chain TAGs. In fact, in figure 1c, where the residual of each individual metabolite beyond the sum of all the considered TAGs (≤56 C and ≤3 double bounds) is plotted, we observed that odd chain TAGs presented a strong inverse association with T2D.

The predictive ability of each lipid score was evaluated using ROC curve analyses, considering age, sex, intervention group, BMI, smoking, leisure time physical activity, hypertension, dyslipidemia and fasting glucose as the basic model to assess the improvement in prediction by adding these groups of lipids. We observed that the sum of all the lipid scores inversely associated with T2D incidence (LP, PC-PL, SM and CE) was able to significantly improve the prediction of T2D beyond that of conventional risk factors, though the size of this improvement was small (AUC excluding lipid scores= 0.82 (CI 95%:0.79-0.85), AUC including LP, PC-PL, SM and CE scores = 0.83 (CI 95%:0.80-0.86); p=0.016 for the comparison).

1-y change lipid scores

One year changes were calculated for each lipid metabolite and for their scores. It is important to note that in these analyses the number of incident cases (only those occurring during after the first year and with available plasma sample) was reduced from 251 to 121 and the statistical power was considerably lower. It is also important that we adjusted all these models for baseline scores and therefore, we were assessing the association of changes beyond baseline predictions. We observed that point estimates for 1-year changes suggested the same direction of the associations observed at baseline: a 1-y increase in LP, PC-PL, SM and CE scores pointed at an inverse association between increased levels of each group of lipids and the subsequent risk of T2D (Table 3S). On the other hand, 1-y increase in TAG, DAG and PE scores suggested a direct association between increased levels of the implicated metabolites and the risk of T2D. However, all these associations did not remain statistically significant (Table 3S). We found a statically significant direct association per each SD for PE score of changes in the model adjusted for PE baseline score, age, sex, intervention group, BMI, smoking, hypertension and dyslipidemia (HR: 1.25; 95% CI: 1.01-1.56, respectively; table 3S).

DISCUSSION

In the framework of the PREDIMED trial, we have identified lipidome (molecular species of lipids) scores for several lipid classes prospectively associated with T2D risk. We found that baseline LP (LPC and LPE), PC-PL, SM and CE were inversely associated with the risk of T2D while baseline TAG, DAG and PE were directly associated with T2D incidence.

When looking at one year changes in these scores, we observed mainly non-significant associations (only PE changes remained significant) but point estimates of the

associations for 1-year changes in scores pointed in the same direction that baseline scores.

It is important to note that T2D cases presented mean baseline high levels of fasting glucose (117±18 mg/dl), probably as a result of a prediabetic-stage in many of them. Thus, we could be working with lipid biomarkers of an established prediabetic profile rather than a diabetes-naïve risk profile. In this context, a recent study published that plasma lipid profiles were similar in both prediabetic and diabetic subjects (6), what may suggest that our identified lipid patterns may be reflecting risk-related profiles for the progression to T2D.

In our study both LPC and LPE, grouped as LP, were associated with reduced risk of T2D incidence. Previous studies found that LPC were reduced in individuals with obesity, insulin resistance and T2D (6,17–19). In fact, increased levels of LPC have been defined as indicators of metabolic health in obesity (20). A potential role in both glucose metabolism and inflammation has been described for LPC, appearing to have glucose lowering and anti-inflammatory effects (20). In the same direction, LPC and LPE were reduced in T2D and associated with the risk of CVD in diabetic patients (21). Similarly to LP, we also found that high levels of PC-PL were related to a lower T2D incidence. Plasmalogens have been widely investigated by their role as endogenous antioxidants, limiting the oxidation of other lipids (7,22). Moreover, other beneficial functions, such as anti-apoptotic and anti-inflammatory have been attributed to them (7) and hence may be decreasing the risk of T2D, as observed in the present study.

The third lipid class inversely associated with T2D risk in our population was the group of SM. In the same line, an association of SM with reduced risk of T2D was reported in the EPIC-Potsdam (23). These results were also reproduced in cognitively healthy T2D subjects (24) and in impaired fasting glucose and T2D metabolomics profiles (25). A

large cohort of prediabetics and diabetic patients also reported an inverse association between plasma odd chain SM and T2D (6), in consistency with our findings. The knockout model of SM synthase results in mitochondrial dysfunction and impaired glucose-stimulated insulin secretion providing a molecular basis to our findings (26).

We found that CEs were associated with a lower risk of T2D. This was an unexpected result taking into account previous cross-sectional studies reporting a strong direct association of CE lipid class with T2D (6,27). However, we found similar results for the association of CEs and CVD in the PREDIMED trial (10). Our hypothesis is that we could be detecting the defined "atherogenic lipoprotein phenotype" in subjects at high T2D risk: high plasma levels of TAG, low levels of HDL and atypically dense LDL particles. In this situation, LDL particles are loaded with TAG instead of CE and after the hydrolysis of TAG in the liver, lipid-depleted LDL particles (small and dense) are released (28). By losing the lipid core, they leave also antioxidant vitamins becoming dense and oxidatively damaged particles that may trigger the foam-cell formation and therefore atherosclerosis. This lipoprotein phenotype has been also suggested for insulin resistance and eventually T2D (29).

We found that DAG, TAG and PE scores were directly and strongly associated with a higher risk of T2D. Higher circulating levels of DAG and short TAGs have been previously associated with T2D (6,30). Thus, our findings confirm this positive association, highlighting the adverse role of short and saturated/low unsaturated species (30). Interestingly, after adjusting the each individual TAG for the total TAG score (sum of all TAGs with ≤56C and ≤3 double bounds), we observed that odd chain TAGs were inversely associated with T2D. Odd chain fatty acids, especially C15:0 and C17:0, have been described as biomarkers of dairy product intake (31) and they have been reported to be associated with a reduced risk of T2D (6,32) and CVD in previous

studies (33). Thus, it seems important to consider the fatty acid content of TAGs to establish plasma risk profiles for T2D.

PEs have been associated with high fasting glucose and T2D (6) and we have confirmed this finding in our population. PEs are minor species in plasma, but they are important structural lipids in membranes, and an increase of PE and an imbalance between PC/PE has been related to obesity and non-alcoholic fatty liver disease (34–36), both conditions related to T2D (6).

The main strengths of our study are: first, the case-cohort design nested in the PREDIMED trial enables the extension of the identified lipid patterns to all PREDIMED participants. Second, the analyses considering the complete lipidome allowed us to observe the effect of each lipid metabolite and each lipid group in the context of coexisting and interacting with the other plasma lipids.

This study also presents some limitations: first, although we have adjusted for several confounding factors, there may be residual confounding by other unknown or unmeasured variables that cannot be controlled. Second, T2D was a secondary endpoint and not the primary endpoint of the PREDIMED trial. Third, our results may not be generalizable to other populations because all study participants lived in a Mediterranean country and were at high cardiovascular risk.

In summary, our results support that before the onset of T2D a plasma lipid profile, characterized by high levels of DAG, short TAG and PE and low levels of LP, PC-PL, SM and CE, could be identified in subjects at high risk for the development of T2D. These results may help to understand the biological mechanisms underlying the link between dyslipidemia and T2D.

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Authors contribution: CR and MAM-G conducted the statistical analyses and drafted the article. CR, FBH, ET, CBC, MR-C, JS-S, and MAM-G made substantial contribution to the conception and design of the work. All authors contributed substantially in the acquisition of data or analysis and interpretation of data.
All authors revised it critically for important intellectual content.
All authors approved the version to be published.

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Figure legends

Figure 1a. HRs per 1-SD in baseline lipid concentrations for lipid groups inversely associated with type 2 diabetes. Lipid species were inverse normally transformed, and HRs were calculated from weighted Cox models adjusted for age, sex, intervention group, BMI, smoking, hypertension, dyslipidemia baseline glucose (linear and quadratic term).

Figure 1b. HRs per 1-SD in baseline lipid concentrations for lipid groups directly associated with type 2 diabetes. Lipid species were inverse normally transformed, and HRs were calculated from weighted Cox models adjusted for age, sex, intervention group, BMI, smoking, hypertension, dyslipidemia baseline glucose (linear and quadratic term).

Figure 1c. HRs per 1-SD for the residual of each triacylglycerol over the total content of the considered triacylglycerol with T2D. Lipid species were inverse normally transformed before calculating the residual, and HRs were calculated from weighted Cox models adjusted for age, sex, intervention group, BMI, smoking, hypertension, dyslipidemia baseline glucose (linear and quadratic term).

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

	Subcohort (n=692)[a]	T2D cases (n=250)
Age	66.5 (5.7)	66.4 (5.7)
Women (%)	63	55.2
BMI	29.9 (3.6)	30.8 (3.3)
Waist circumference (cm)	100 (11)	103 (10)
LTPA (METS-min/day)	238 (238)	249 (234)
Fasting glucose (mg/dl)	98 (14)	117 (18)
HDL-cholesterol (mg/dl)	56.9 (14.2)	52.8 (11.6)
LDL-cholesterol (mg/dl)	138.3 (30.5)	135.0 (30.2)
Total colesterol (mg/dl)	219.9 (35.6)	218.4 (39.1)
Triglycerides (mg/dl)	129.8 (92.2-149.3)[b]	160.9 (109-180)[b]
Dyslipidemia (%)	85	80
Hypertension (%)	91	96
Smoking		
Non-smoker (%)	61	53
Current smoker (%)	16	25
Former smoker (%)	23	22
Total energy intake (Kcal/day)	2276 (564)	2321 (616)
Adherence to MedDiet	8.6 (2.0)	8.4 (2.0)
Intervention group (%)		
Control (%)	32	36
MedDiet+EVOO (%)	31	30
MedDiet+nuts (%)	37	34

[a]Including 53 overlapping cases

[b] Interquartile range

T2D: type 2 diabetes; LTPA: Leisure time physical activity; MedDiet: Mediterranean diet; EVOO: extra-virgin olive oil

Table 2. Association (hazard ratios, 95% confidence intervals) between baseline lipid factors (PCA extracted) and T2D risk (adjusted for age, sex and intervention group)

	Quartiles of factors				Linear trend	Per SD
	Q1	**Q2**	**Q3**	**Q4**		
Factor 3*	Ref.	0.62 (0.37-1.05)	0.79 (0.47-1.32)	**0.45 (0.26-0.79)**	**0.017**	**0.70 (0.57-0.85)**
Factor 7*	Ref.	**0.62 (0.39-0.97)**	**0.39 (0.23-0.67)**	**0.36 (0.20-0.63)**	**<0.001**	**0.58 (0.49-0.70)**
Factor10*	Ref.	0.80 (0.48-1.33)	0.48 (0.28-0.81)	**0.56 (0.33-0.95)**	**0.018**	**0.74 (0.62-0.88)**
Factor13*	Ref.	1.00 (0.61-1.66)	0.78 (0.47-1.32)	**0.58 (0.34-1.00)**	0.146	0.93 (0.78-1.10)
Factor 1*	Ref.	1.39 (0.77-2.52)	**2.24 (1.29-3.88)**	**2.72 (1.59-4.66)**	**<0.001**	**1.62 (1.36-1.92)**
Factor 5*	Ref.	1.44 (0.83-2.45)	1.14 (0.65-2.00)	**2.22 (1.31-3.77)**	**0.030**	**1.24 (1.04-1.47)**
Factor 11*	Ref.	1.25 (0.72-2.18)	1.78 (1.03-3.10)	**2.02 (1.15-3.54)**	**0.011**	1.18 (0.99-1.40)

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

Table 3. Association (hazard ratios, 95% confidence intervals) between baseline lipid scores and T2D risk.

	Quartiles of scores				Linear trend	Per SD
	Q1	**Q2**	**Q3**	**Q4**		
Lysophospholipids (LP) score (lysophosphatidylcholines and lysophosphatidylethanolamines); n of molecules=18						
M1*	Ref.	0.82 (0.56-1.19)	**0.49 (0.32-0.75)**	**0.46 (0.29-0.71)**	**<0.001**	**0.73 (0.63-0.85)**
M2*	Ref.	0.86 (0.58-1.27)	**0.48 (0.31-0.76)**	**0.51 (0.32-0.81)**	**<0.001**	**0.74 (0.63-0.87)**
M3*	Ref.	1.08 (0.63-1.85)	**0.48 (0.23-0.99)**	0.68 (0.37-1.25)	0.053	0.81 (0.65-1.02)
Phosphatidylcholines plasmalogens (PC-pl) score ; n of molecules=15						
M1*	Ref.	0.78 (0.53-1.17)	0.77 (0.52-1.16)	**0.36 (0.22-0.58)**	**<0.001**	**0.78 (0.68-0.90)**
M2*	Ref.	0.80 (0.53-1.21)	0.81 (0.54-1.22)	**0.38 (0.23-0.64)**	**0.002**	**0.82 (0.71-0.95)**
M3*	Ref.	**0.63 (0.34-1.14)**	0.67 (0.37-1.23)	**0.31 (0.15-0.66)**	**0.004**	**0.76 (0.60-0.96)**
Sphingomielines (SM) score; n of molecules=11						
M1*	Ref.	**0.49 (0.32-0.75)**	**0.58 (0.38-0.89)**	**0.32 (0.19-0.54)**	**<0.001**	**0.67 (0.56-0.80)**
M2*	Ref.	**0.46 (0.30-0.72)**	**0.56 (0.35-0.87)**	**0.31 (0.18-0.52)**	**<0.001**	**0.69 (0.57-0.83)**
M3*	Ref.	**0.29 (0.15-0.57)**	**0.49 (0.25-0.94)**	**0.25 (0.11-0.55)**	**0.001**	**0.67 (0.51-0.88)**
Cholesterol esters (CE) score; n of molecules=13						
M1*	Ref.	0.76 (0.50-1.14)	0.64 (0.43-0.95)	**0.34 (0.21-0.54)**	**<0.001**	**0.68 (0.58-0.79)**
M2*	Ref.	0.87 (0.56-1.35)	0.74 (0.49-1.12)	**0.39 (0.24-0.65)**	**0.003**	**0.70 (0.59-0.84)**
M3*	Ref.	0.56 (0.28-1.12)	0.70 (0.38-1.29)	**0.35 (0.17-0.73)**	**0.016**	**0.66 (0.51-0.85)**
Triacylglycerides (TAG) score (≤56C and ≤3 double bonds); n of molecules=40						
M1*	Ref.	**1.73 (1.06-2.83)**	**2.18 (1.35-3.51)**	**2.94 (1.85-4.67)**	**<0.001**	**1.49 (1.27-1.75)**
M2*	Ref.	**1.77 (1.06-2.94)**	**2.10 (1.28-3.43)**	**2.55 (1.58-4.11)**	**<0.001**	**1.39 (1.18-1.64)**
M3*	Ref.	1.54 (0.71-3.33)	2.07 (0.98-4.37)	1.94 (0.93-4.07)	0.095	**1.29 (1.00-1.66)**
Diacylglycerides (DAG) score; n of molecules=14						
M1*	Ref.	1.14 (0.70-1.86)	**2.13 (1.35-3.36)**	**2.76 (1.77-4.29)**	**<0.001**	**1.58 (1.33-1.86)**
M2*	Ref.	1.25 (0.75-2.08)	**1.95 (1.21-3.14)**	**2.46 (1.56-3.88)**	**<0.001**	**1.48 (1.24-1.77)**
M3*	Ref.	1.22 (0.57-2.62)	1.72 (0.88-3.36)	**2.03 (1.08-3.81)**	**0.017**	**1.35 (1.05-1.72)**
Phosphatidylethanolamines (PE) score; n of molecules=12						
M1*	Ref.	1.51 (0.95-2.39)	1.57 (0.95-2.39)	**2.55 (1.64-3.95)**	**<0.001**	**1.45 (1.23-1.70)**
M2*	Ref.	1.43 (0.89-2.31)	1.48 (0.92-2.38)	**2.13 (1.35-3.35)**	**0.002**	**1.35 (1.15-1.59)**
M3*	Ref.	1.46 (0.77-2.75)	0.72 (0.36-1.46)	1.33 (0.72-2.49)	0.485	1.21 (0.96-1.53)

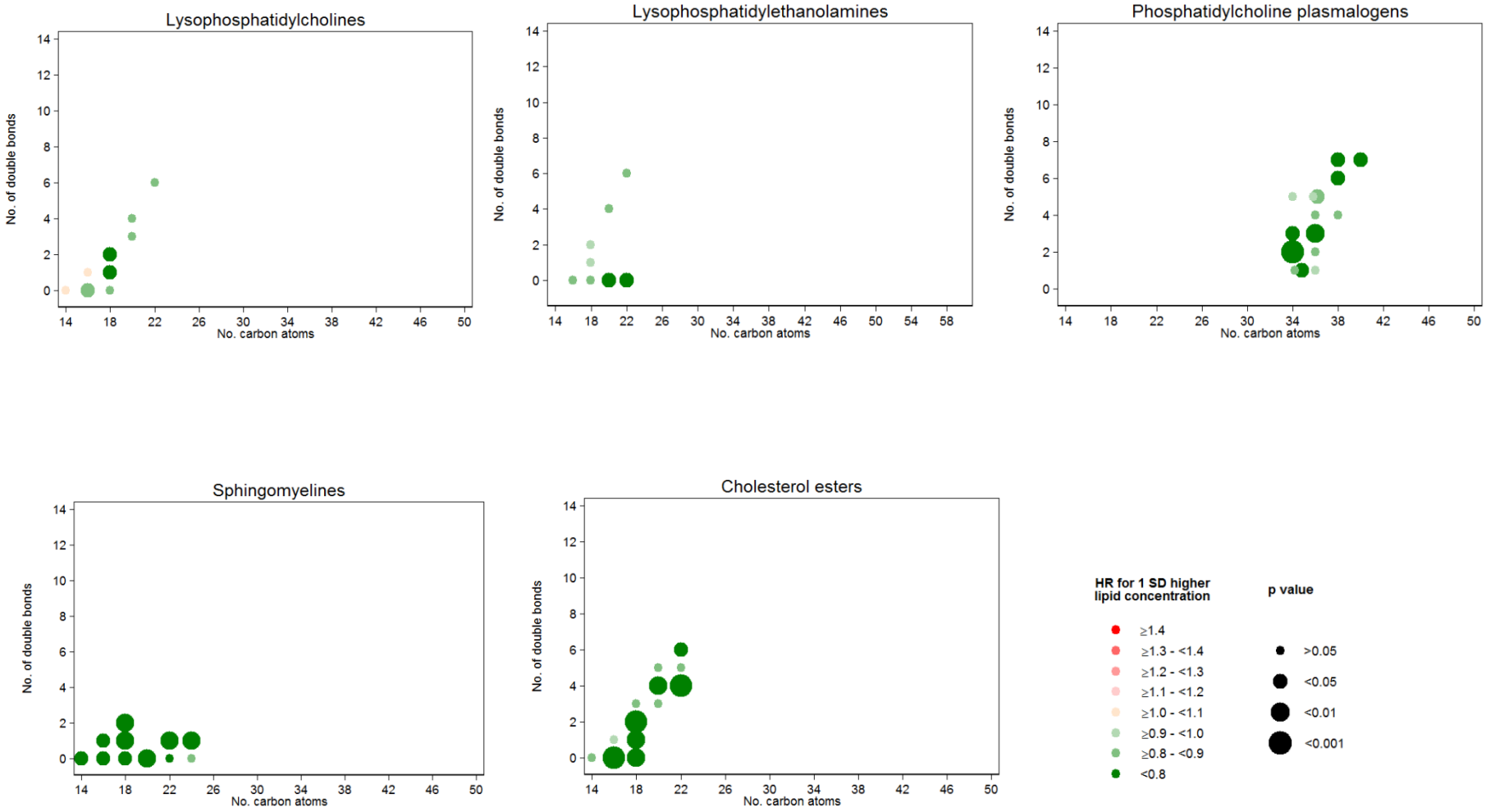

Lysophosphatidylcholines
Lysophosphatidylethanolamines
Phosphatidylcholine plasmalogens
Sphingomyelines
Cholesterol esters
No. of double bonds
No. carbon atoms
HR for 1 SD higher lipid concentration
≥1.4
≥1.3 - <1.4
≥1.2 - <1.3
≥1.1 - <1.2
≥1.0 - <1.1
≥0.9 - <1.0
≥0.8 - <0.9
<0.8
p value
>0.05
<0.05
<0.01
<0.001

Figure 1a

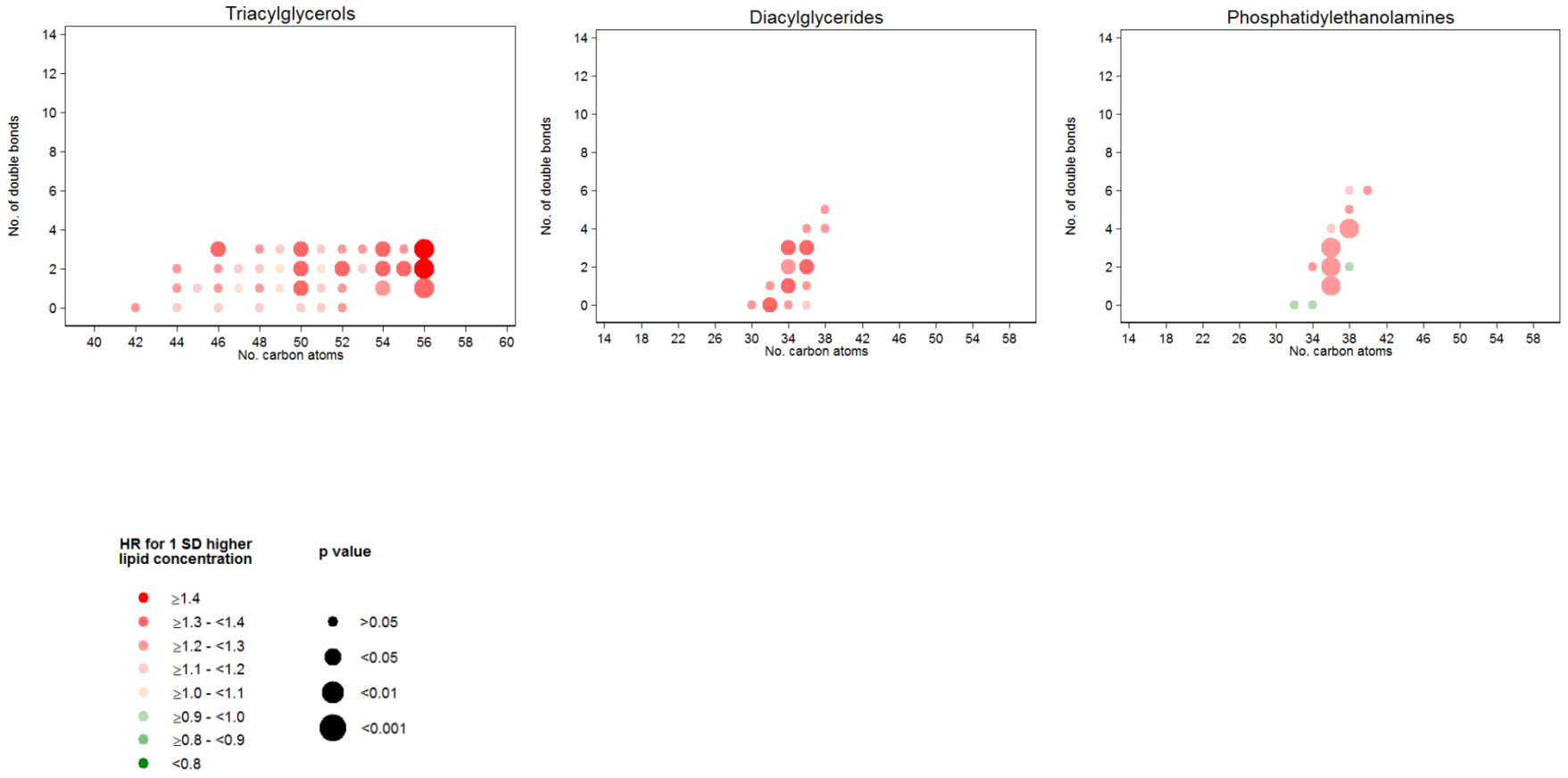

Triacylglycerols
Diacylglycerides
Phosphatidylethanolamines
No. of double bonds
No. carbon atoms
HR for 1 SD higher lipid concentration
≥1.4
≥1.3 - <1.4
≥1.2 - <1.3
≥1.1 - <1.2
≥1.0 - <1.1
≥0.9 - <1.0
≥0.8 - <0.9
<0.8
p value
>0.05
<0.05
<0.01
<0.001

Figure 1b

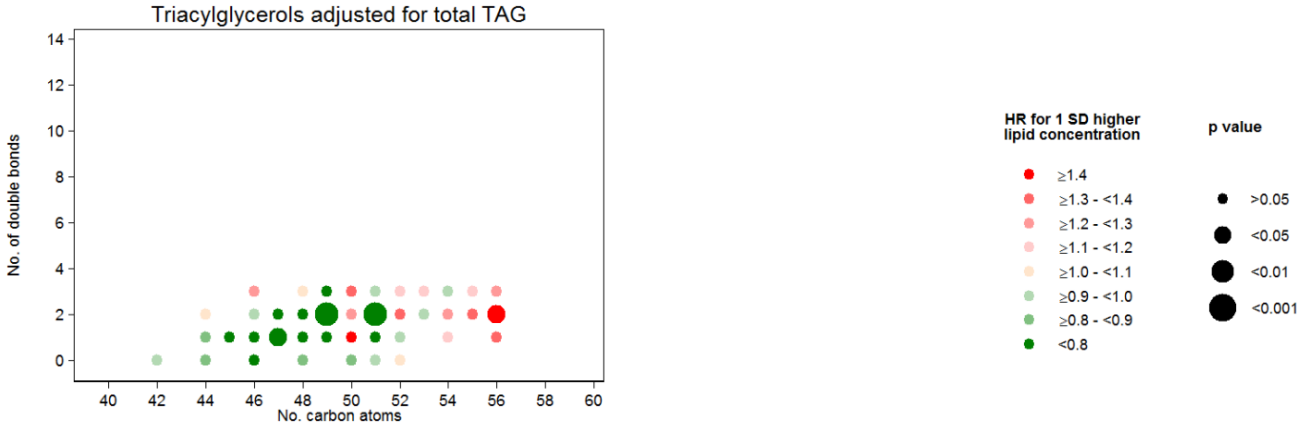
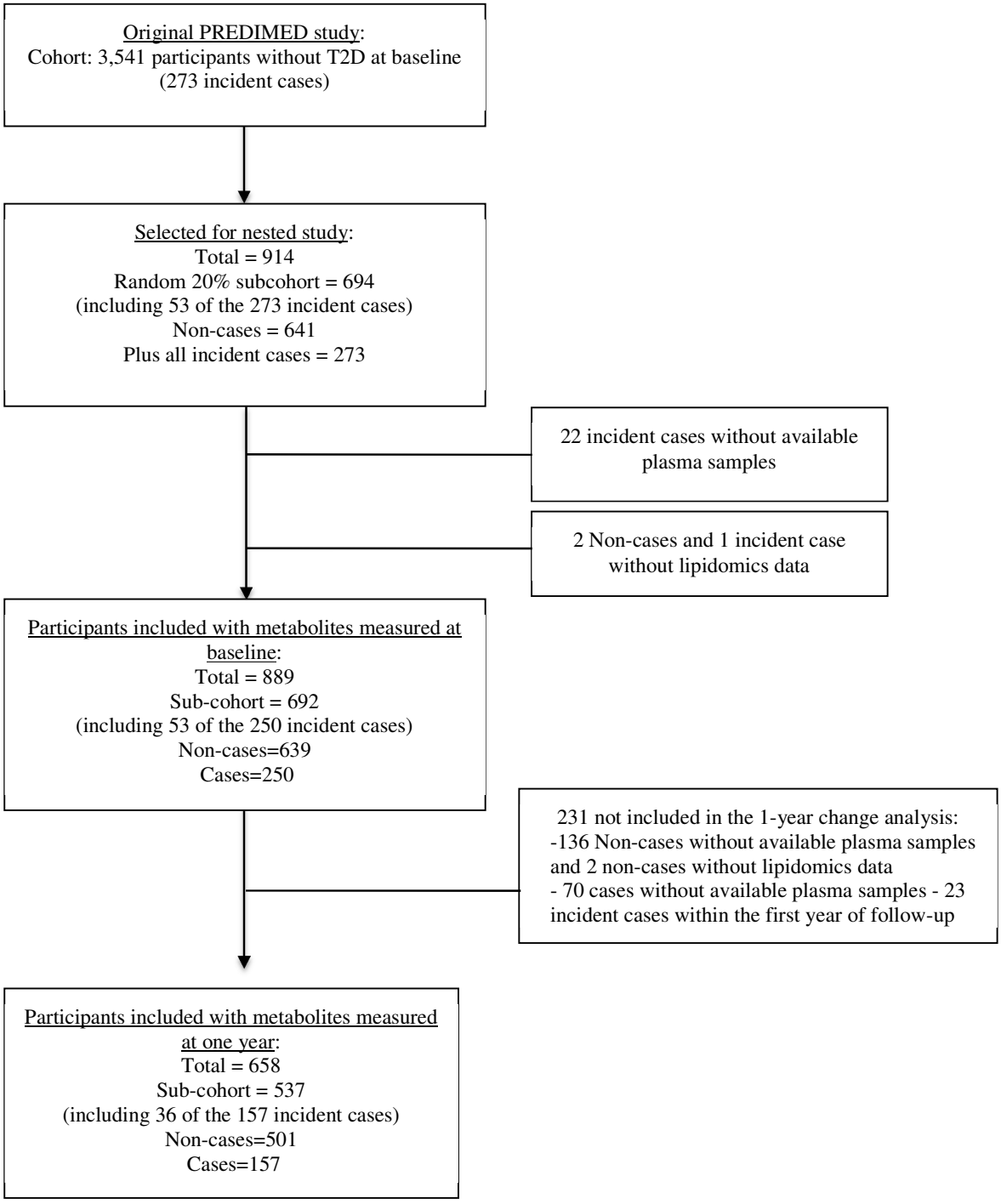

Triacylglycerols adjusted for total TAG
No. of double bonds
No. carbon atoms
0
2
4
6
8
10
12
14
40
42
44
46
48
50
52
54
56
58
60
HR for 1 SD higher lipid concentration
≥1.4
≥1.3 - <1.4
≥1.2 - <1.3
≥1.1 - <1.2
≥1.0 - <1.1
≥0.9 - <1.0
≥0.8 - <0.9
<0.8
p value
>0.05
<0.05
<0.01
<0.001

Figure 1c

SUPPLEMENTAL MATERIAL

Figure 1. Flow-chart of the case-cohort design



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 (2 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.

Table 1S. Eigenvalues and variance explained by the 15 extracted factors (PCA).

Factor	Eigenvalue	Variance explained (%)	Cumulative
Factor 1	53.35	25.77	25.77
Factor 2	31.76	15.35	41.12
Factor 3	23.83	11.51	52.63
Factor 4	14.25	6.88	59.51
Factor 5	9.94	4.8	64.32
Factor 6	8.33	4.03	68.34
Factor 7	6.38	3.08	71.42
Factor 8	5.88	2.84	74.26
Factor 9	4.70	2.27	76.54
Factor 10	3.77	1.82	78.36
Factor 11	2.91	1.41	79.76
Factor 12	2.53	1.22	80.99
Factor 13	2.43	1.17	82.16
Factor 14	2.19	1.06	83.22
Factor 15	2.00	0.97	84.19

Table 2S. Association between baseline lipid factors (PCA extracted) and T2D risk (adjusted for age, sex and intervention group)

	Factor	Description	Components
Inverse association with T2D	**3**	PC-plasmalogens Sphingomyelines (SM) Lysophosphatidylcholines (LPC) and Lysophosphatidylethanolamines (LPE)	364pca 182ce 386pcplasmalogen 160ce 407pcplasmalogen 241ceramided181 343pc 382pc 364pcplasmalogen 343pcplasmalogen 362pcplasmalogen 341pcplasmalogenb 363pc 361psplasmalogen 384pcplasmalogen 341pcplasmalogena 361pcplasmalogen 342pcplasmalogen 360pc 320pc 363pcplasmalogen 382pe 342pc 362pc 340pc 362psplasmalogen 220ceramided181 240ceramided181 5410tag 180ce 160ceramided181 182sm 140sm 241sm 181sm 240sm cholesterol 161sm 180sm 221sm 200sm 160sm 220sm
	7	Cholesterol esters (CE)	c340pc 181lpe 140lpc 226lpe 204lpe 182lpc 205lpc 161lpc 204lpc c226lpc 160lpe 220lpe 200lpe 180lpe 181lpc 180lpc 203lpc 160lpc
	10		406ps 361peplasmalogen 200lpe 513tag 340pe 532tag 533tag 553tag 382pe
	13		361psplasmalogen 320pc 342pcplasmalogen 341pcplasmalogena 364pcplasmalogen
Direct association with T2D	**1**	Triacylglycerides (TAG) with≤56 C and ≤3 double bonds Diacylglycerides (DAG) Phosphatidylethanolamines (PE)	182ce* 224ce* 160ce* 226ce* 204ce* 181ce* 382pc 161lpc 361pc 320pc 384dag 406pe 362dag 385dag 161ce 341pc 140ce 527tag 542tag 526tag 364pe 383pc 532tag 513tag 562tag 343pc 362pe 406ps 361pe 342pe 343dag 344pc 340pe 522tag 552tag 322pc 504tag 342dag 505tag 140lpc 320pe 341dag 340dag 561tag 361dag 321pc 503tag 484tag 541tag 512tag 300pc 301pc 493tag 300dag 521tag 321dag 520tag 502tag 510tag 463tag 483tag 320dag 501tag 492tag 442tag 420tag 491tag 511tag 451tag 471tag 500tag 480tag 472tag 441tag 462tag 482tag 460tag 440tag 481tag 461tag
	5		160ce 533tag 341pc 341dag 541tag 361dag 544tag 523tag 361pc 241ceramided181 361pe 532tag 564tag 552tag 522tag 553tag 562tag 362dag 542tag 563tag 543tag
	11		406pe 362pe 384pe 385pe 363pe 386pe 342pe 364pe

*Negative loading factors

Table 3S. Association (hazard ratios, 95% confidence intervals) between 1-y changes lipid scores and T2D risk

	Quartiles of scores				Linear trend	Per SD
	Q1	Q2	Q3	Q4		
Lysophospholipids (LP) score of changes (lysophosphatidylcholines and lysophosphatidylethanolamines); n of molecules=18						
M1*	Ref.	0.90 (0.50-1.60)	0.79 (0.44-1.43)	0.77 (0.42-1.43)	0.350	0.88 (0.70-1.11)
M2*	Ref.	0.97 (0.53-1.77)	0.90 (0.49-1.66)	0.75 (0.38-1.47)	0.299	0.87 (0.68-1.11)
M3*	Ref.	2.43 (1.07-5.50)	0.84 (0.35-2.03)	1.18 (0.47-2.97)	0.852	0.94 (0.69-1.30)
Phosphatidylcholines plasmalogens score of changes (phosphatidylcholines-pl); n of molecules=15						
M1*	Ref.	0.64 (0.37-1.11)	0.61 (0.34-1.07)	0.57 (0.32-1.03)	0.079	0.88 (0.72-1.07)
M2*	Ref.	0.62 (0.35-1.12)	0.63 (0.35-1.13)	0.57 (0.31-1.05)	0.102	0.91 (0.75-1.11)
M3*	Ref.	1.15 (0.50-2.65)	1.14 (0.46-2.79)	0.64 (0.24-1.70)	0.402	0.88 (0.64-1.22)
Sphingomielines (SM) score of changes; n of molecules=11						
M1*	Ref.	0.95 (0.54-1.70)	0.93 (0.52-1.64)	0.68 (0.36-1.27)	0.254	0.86 (0.70-1.06)
M2*	Ref.	1.09 (0.60-1.99)	0.98 (0.53-1.80)	0.74 (0.39-1.44)	0.330	0.89 (0.71-1.11)
M3*	Ref.	1.18 (0.49-2.88)	1.77 (0.75-4.17)	0.76 (0.31-1.87)	0.900	0.92 (0.69-1.23)
Cholesterol esters (CE) score of changes ; n of molecules=13						
M1*	Ref.	0.81 (0.47-1.40)	0.73 (0.42-1.28)	0.84 (0.47-1.49)	0.518	0.82 (0.65-1.04)
M2*	Ref.	0.88 (0.50-1.56)	0.81 (0.45-1.45)	0.92 (0.51-1.68)	0.901	0.85 (0.67-1.08)
M3*	Ref.	1.19 (0.55-2.58)	0.82 (0.31-2.20)	1.15 (0.47-2.85)	0.829	0.88 (0.60-1.28)
Triacylglycerides (TAG) score of changes (≤56C and ≤3 double bonds); n of molecules=40						
M1*	Ref.	0.83 (0.47-1.47)	1.08 (0.60-1.97)	1.52 (0.86-2.68)	0.127	1.19 (0.96-1.46)
M2*	Ref.	0.83 (0.47-1.49)	1.10 (0.60-2.02)	1.40 (0.76-2.60)	0.263	1.19 (0.97-1.47)
M3*	Ref.	0.93 (0.40-2.17)	1.22 (0.52-2.87)	1.48 (0.58-3.78)	0.381	1.24 (0.92-1.68)
Diacylglycerides (DAG) score of changes; n of molecules=14						
M1*	Ref.	1.33 (0.76-2.32)	1.04 (0.60-1.82)	1.49 (0.85-2.64)	0.302	1.19 (0.98-1.45)
M2*	Ref.	1.27 (0.71-2.25)	1.10 (0.61-1.95)	1.39 (0.77-2.52)	0.439	1.15 (0.94-1.40)
M3*	Ref.	2.19 (0.92-5.20)	1.98 (0.80-4.89)	1.69 (0.68-4.27)	0.286	1.20 (0.91-1.57)
Phosphatidylethanolamines (PE) score of changes; n of molecules=12						
M1*	Ref.	1.21 (0.69-2.11)	1.23 (0.68-2.22)	**1.87 (1.06-3.30)**	0.060	**1.25 (1.02-1.54)**
M2*	Ref.	1.23 (0.70-2.18)	1.29 (0.71-2.36)	**1.85 (1.01-3.38)**	0.100	**1.25 (1.01-1.56)**
M3*	Ref.	1.39 (0.62-3.15)	1.80 (0.73-4.43)	1.76 (0.69-4.47)	0.167	1.27 (0.93-1.73)