SUPPLEMENTAL MATERIAL

An unbiased lipid phenotyping approach to study the genetic determinants and mechanisms of coronary heart disease risk factors

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Appendix 1. Supplemental Methods

Lipidomics sample extraction. An automated method for the extraction of lipids was developed using an Anachem Flexus automated liquid handler (Anachem, Milton Keynes, UK). Eighty PROMIS samples, four blanks, and 12 QC samples in 1.2 mL Cryovials were placed on the Flexus, then 100 µL of MilliQ H_2O was added to each of the wells and mixed, and then 100 µL of the mixture was transferred to a glass coated 2.4 mL deep well plate (Plate+™, Esslab, Hadleigh, UK). Next, 250 µL of MeOH was added containing six internal standards (0.6 µM 1,2-di-O-octadecyl-*sn*-glycero-3-phosphocholine, 1.2 µM 1,2-di-*O*-phytanyl-*sn*-glycero-3-phosphoethanolamine, 0.6 µM C8-cerramide, 0.6 µM *N*-heptadecanoyl-D-*erythro*-sphingosylphosporylcholine, 6.2 µM undecanoic acid, 0.6 µM trilaurin), followed by 500 µL of methyl *tert*-butyl ether (MTBE). The plates were then sealed (using Corning aluminum micro-plate sealing tape [Sigma Aldrich Company, UK]) and shaken for 10 min at 600 rpm, after which the plate was transferred to a centrifuge and spun for 10 min at 6000 rpm. Each well in the resulting plate had two layers, with an aqueous layer at the bottom and an organic layer on top. A 96-head micro-dispenser (Hydra Matrix, Thermo Fisher Ltd, Hemel Hampstead, UK) was used to transfer 25 µL of the organic layer to a glass coated 240 µL low well plate (Plate+™, Esslab, Hadleigh, UK), and 90 µL of MS-mix (7.5 mM NH_4Ac IPA:MeOH [2:1]) was added using a Hydra Matrix, after which the plate was sealed and stored at –20 °C until analysis.

Direct infusion high-resolution mass spectrometry. All samples were infused into an Exactive Orbitrap (Thermo, Hemel Hampstead, UK), using a Triversa Nanomate (Advion, Ithaca, US). The Nanomate infusion mandrel was used to pierce the seal of each well before analysis, after which, with a fresh tip, 5 µL of sample was aspirated, followed by an air gap (1.5 µL). The tip was pressed against a fresh nozzle and the sample was dispensed using 0.2 psi nitrogen pressure. Ionization was achieved with a 1.2 kV voltage. The Exactive started acquiring data 20 seconds after sample aspiration began. The Exactive acquired data with a scan rate of 1 Hz (resulting in a mass resolution of 65,000 full width at half maximum [fwhm] at 400 *m/z*). After 72 seconds of acquisition in positive mode

the Nanomate and the Exactive switched over to negative mode, decreasing the voltage to -1.5 kV. The spray was maintained for another 66 seconds, after which the analysis was stopped and the tip discarded, before the analysis of the next sample began. The sample plate was kept at 15 °C throughout the analysis. Samples were run in row order and repeated multiple times if necessary to ensure accuracy.

Principal component analysis. PCA was conducted on the normalized relative intensities of the lipids, with orthogonal rotation of the matrix loadings, and the first four principal components were retained (eigenvalues >3.6, explaining 69% of the covariance in the relative intensities of the lipids). Lipids with more than 10% missing data were excluded from the analysis.

Logistic regression. Unconditional logistic regression models adjusting for age and sex were used to assess the association of the four principal components with several established CHD risk factors—overweight, obesity, hypertension, and diabetes. CHD risk factors were defined using well-established cut-off points from published guidelines.[1-4] Hypertension was defined as SBP ≥ 140 mm Hg or DBP ≥ 90 mm Hg; overweight and obese were defined as body mass index (BMI) ≥ 25 kg/m^2 or BMI ≥ 30 kg/m^2, respectively; and diabetes was defined as either FPG ≥ 126 mg/dL or HbA_{1c} ≥ 6.5%.

Genetic analyses. Linear regression was used to determine the association of each principal component with genotyped and imputed single nucleotide polymorphisms (SNPs) using SNPTEST v2.4.1,[5] which was performed separately for the samples genotyped on each of the two genetic platforms. Residuals were calculated from the null model for each lipid, which included adjustment for age group, sex, date of survey, plate (batch), and fasting status. To account for population stratification and genetic substructure in the data, PCA analyses were conducted on the multi-dimensional scaling matrix created from autosomal SNPs as implemented in PLINK; the first six principal components were subsequently added to each model. A missing data likelihood score test was used when testing for

association at imputed SNPs to account for genotype uncertainty. Beta estimates and standard errors from the association results for the two genetic platforms were combined in a fixed-effect inverse-variance-weighted meta-analysis using the latest version of METAL (2011-03-25).[6] For each lipid, Q-Q plots of the results were constructed and inflation factors were checked to ensure that the values were not too extreme ($\lambda < 1.05$). A conservative Bonferroni correction was then applied for testing multiple lipids, using PCA to determine the number of components (56 in this case) that explained at least 95% of the variance, as has been recently proposed as appropriate for metabolomics studies with correlated traits.[7] A genome-wide significance threshold of $P < 8.9 \times 10^{-10}$ ($5 \times 10^{-8} / 56$) was therefore used. Summary results were compiled for any SNPs with $-\log_{10}$ *P*-values less than the corresponding Bonferroni-corrected *P*-values.

Technical discussion on DIHRMS: Signals were annotated to a molecular formula on the basis of the accurate mass. The resolution of 65,000 at 400 *m/z,* as used in this study, allowed for the baseline separation of, for instance, molecular formulae $C_{41}H_{78}NPO_8+H^+$ (*m/z* 744.554) and $C_{42}H_{82}NPO_7+H^+$ (*m/z* 744.590), but was unable to determine if the former was PC(33:2) or PE(36:2) as the species are isobaric. The average mass accuracy error in the measurement of the *m/z* was 0.85 ppm across all intact lipids, with the highest difference for detected lipids of 2.69 ppm for PC ae (37:4). However, we want to highlight that this did not mean that only one lipid species contribyuted to a specific mass-to-charge ratio. For example, the ion which we identified as TG(52:2) with *m/z* 876.802 could be many different triglyceride lipids [e.g. TG(16:0/18:2/18:0), TG(14:0/16:0/22:2) or TG(16:0/18:1/18:1)], which all have the same molecular weight. Interpretation of species at the chemical formula level allows us to model changes within lipid pools according to biological processes such as chain elongation and desaturation. We assume that a given signal peak was likely to be a combination of several lipid species. Our annotation was further based on fragmentation data for the most predominant ion through additional LC-MS/MS analyses.

Because the "open-profiling" approach did not predetermine which lipid species would be detected, it provided data on all ionizable molecules and was therefore very sensitive to contamination, especially of compounds with high ionization efficiencies. In all analyses, we found adipates (*m/z* 371.316) and organophosphates, such as Tris(ditert-butylphenyl)phosphite (*m/z* 647.459) and its oxidation products (*m/z* 663.454), that leached from plastics into the organic solvents. However, using glass-coated well-plates minimized the contact time of the samples with the plastics, and by using blanks and QCs at three different concentrations, we were able to exclude the contaminating ions (approximately half of all the signals) from the final data set. The use of glass-coated well plates was essential to obtain both precise and reliable data. Furthermore, as the method relied on nanoflow, contaminants had minimal impact on the ionization efficiency.

The developed peak-picking algorithm enabled us to process the almost seven thousand data files using paralellization with a processing time of about four minutes per sample per core. The analysis time was greatly sped up by processing each file independently since there was no requirement to load all files jointly into memory to perform the alignment; the ability to perform analyses in parallel also greatly sped up the analysis time. This approach is only suitable to compare similar samples where the same lipids are expected, as it requires known lipids with their target *m/z*. The results required manual curation as in certain cases the target *m/z* was too close to an isotope or adduct of another lipid. We therefore confirmed the identity of all the ions that passed the QC filter, and selected samples were analyzed by high-resolution LC-MS/MS to confirm lipid annotations.

References for Supplemental Methods

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Supplemental Tables

eTable 1. Demographic and clinical characteristics and coronary heart disease risk factors of PROMIS participants and Pakistanis

	PROMIS controls assayed by DIHRMS (n=5,551)		All PROMIS controls (n=18,564)		DHS Pakistan (n=13,558)	
Variable	**No. of subjects**	**Mean (SD) or %**	**No. of subjects**	**Mean (SD) or %**	**No. of subjects**	**Mean (SD) or %**
Anthropometric markers						
Age at survey (yrs)	5,511	54 (9)	18,564	56 (9)	13,558	33 (9)
Body-mass index (kg/m^2)	5,412	26 (5)	18,290	26 (5)	4,698	25 (6)
Waist-to-hip ratio	5,441	0.96 (0.12)	18,344	0.95 (0.06)	-	-
Systolic blood pressure (mm Hg)	5,438	128 (17)	18,255	128 (17)	-	-
Diastolic blood pressure (mm Hg)	5,435	81 (9)	18,247	81 (10)	-	-
Circulating lipid biomarkers						
Total cholesterol (mmol/L)	5,392	4.63 (1.34)	17,935	4.68 (1.30)	-	-
HDL cholesterol (mmol/L)	5,380	0.89 (0.27)	17,881	0.93 (0.28)	-	-
LDL cholesterol (mmol/L)	5,313	2.77 (1.03)	17,491	2.81 (1.01)	-	-
Non-HDL cholesterol (mmol/L)	5,380	3.74 (1.31)	17,884	3.75 (1.27)	-	-
Log$_e$ triglycerides (mmol/L)	5,387	0.74 (0.53)	17,920	0.69 (0.53)	-	-
Categorical variables						
Sex	5,511		18,564		13,558	
Male	4,352	79%	14,049	76%	12,409	92%
Female	1,159	21%	4,515	24%	1,149	8%
Tobacco consumption status	5,500		18,512		13,542	
Not current	3,817	69%	13,218	71%	12,256	93%
Current	1,683	31%	5,294	29%	1,016	8%
History of diabetes	5,500		18,516		-	
No	4,739	86%	16,081	87%	-	-
Yes	761	14%	2,435	13%	-	-
CHD risk factors						
Overweight	5,412		18,290		4,698	
No	2,381	44%	7,830	43%	2,807	60%
Yes	3,031	56%	10,460	57%	1,891	40%
Obese	5,412		18,290		4,698	
No	4,517	83%	15,339	84%	4,031	86%
Yes	895	17%	2,951	16%	667	14%
Hypertension	5,438		18,257		-	
No	4,479	82%	15,017	83%	-	-
Yes	959	18%	3,240	18%	-	-
Diabetes	4,100		8,503		-	
No	2,525	62%	5,500	65%	-	-
Yes	1,575	38%	3,003	35%	-	-

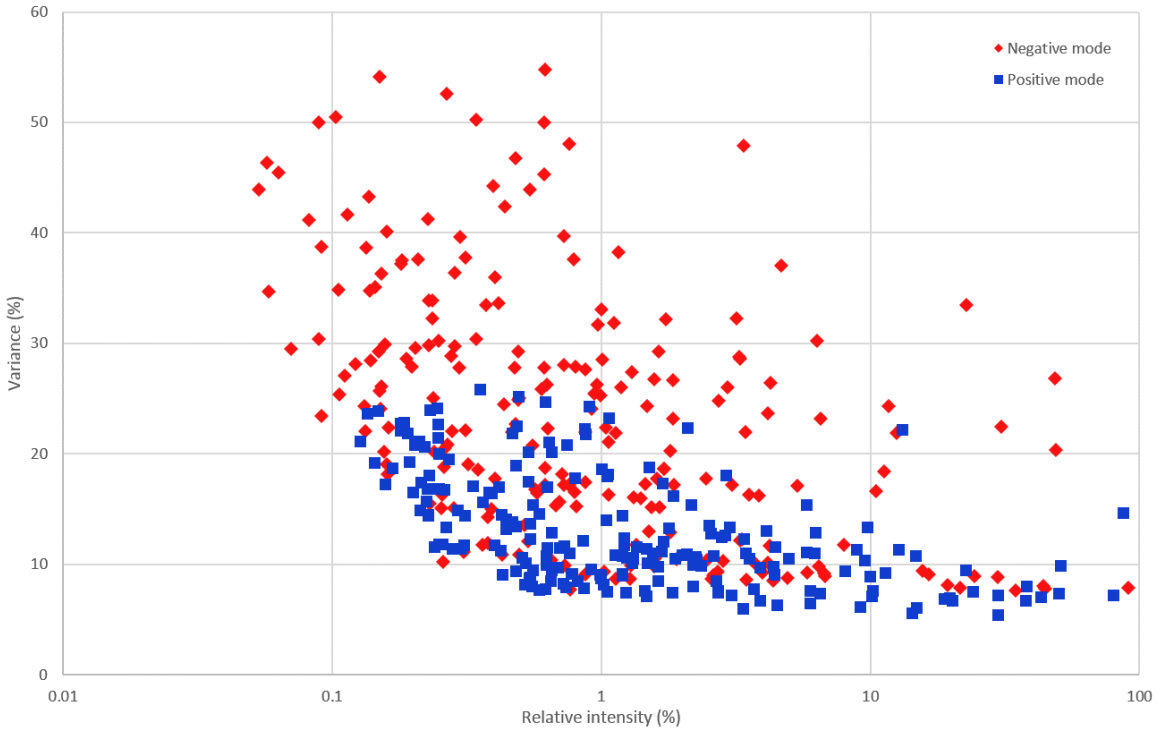
Abbreviations: BMI = body mass index; **CHD** = coronary heart disease; **DBP** = diastolic blood pressure; **DHS** = Demographic & Health Surveys; **FPG** = fasting plasma glucose; **SBP** = systolic blood pressure. **Definitions: Diabetes** = HbA_{1c} ≥ 6.5%; **Hypertension** = SBP ≥ 140 mm Hg or DBP ≥ 90 mm Hg; **Obese** = BMI ≥ 30 kg/m^2; **Overweight** = BMI ≥ 25 kg/m^2. **Note:** Percentages may not add up to 100% due to rounding. Data for the overall Pakistani population was obtained from the DHS.[28] A dash indicates that data are not available.

eTable 2. Summary of associations of principal components of lipids with lead SNP in each locus

Principal component	rsID	Chr:Pos (GRCh37)	Effect allele	Other allele	Effect	SE	*P*-value	Locus
Second	rs662799	chr11:116663707	A	G	-0.9963	0.1819	4.32E-08	*APOA5-APOC3*
Third	rs651821	chr11:116662579	T	C	1.2532	0.1647	2.74E-14	*APOA5-APOC3*
Fourth	rs174568	chr11:615693816	T	C	-1.0686	0.1582	1.44E-11	*FADS1-2-3*
Fourth	rs964184	chr11:116648917	C	G	1.2218	0.1428	1.17E-17	*APOA5-APOC3*

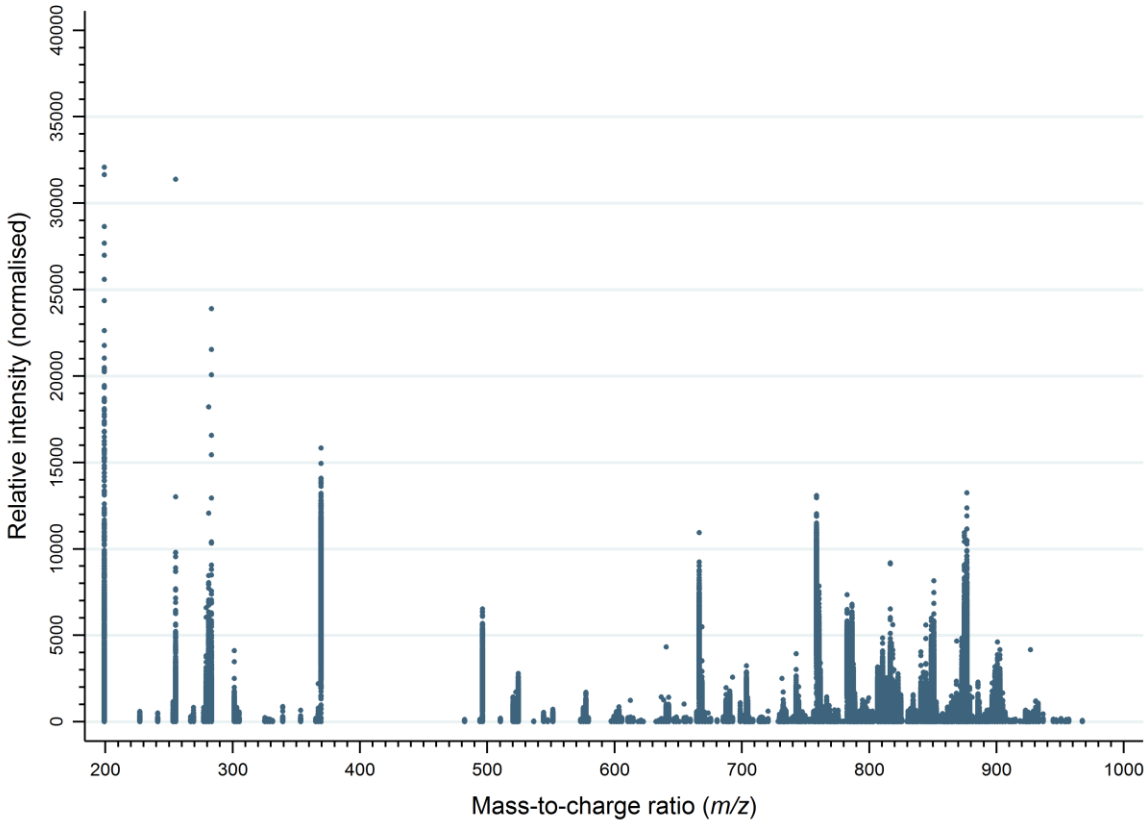
Supplemental Figures

eFigure 1. Coefficient of variation for lipids in positive and negative ionization modes

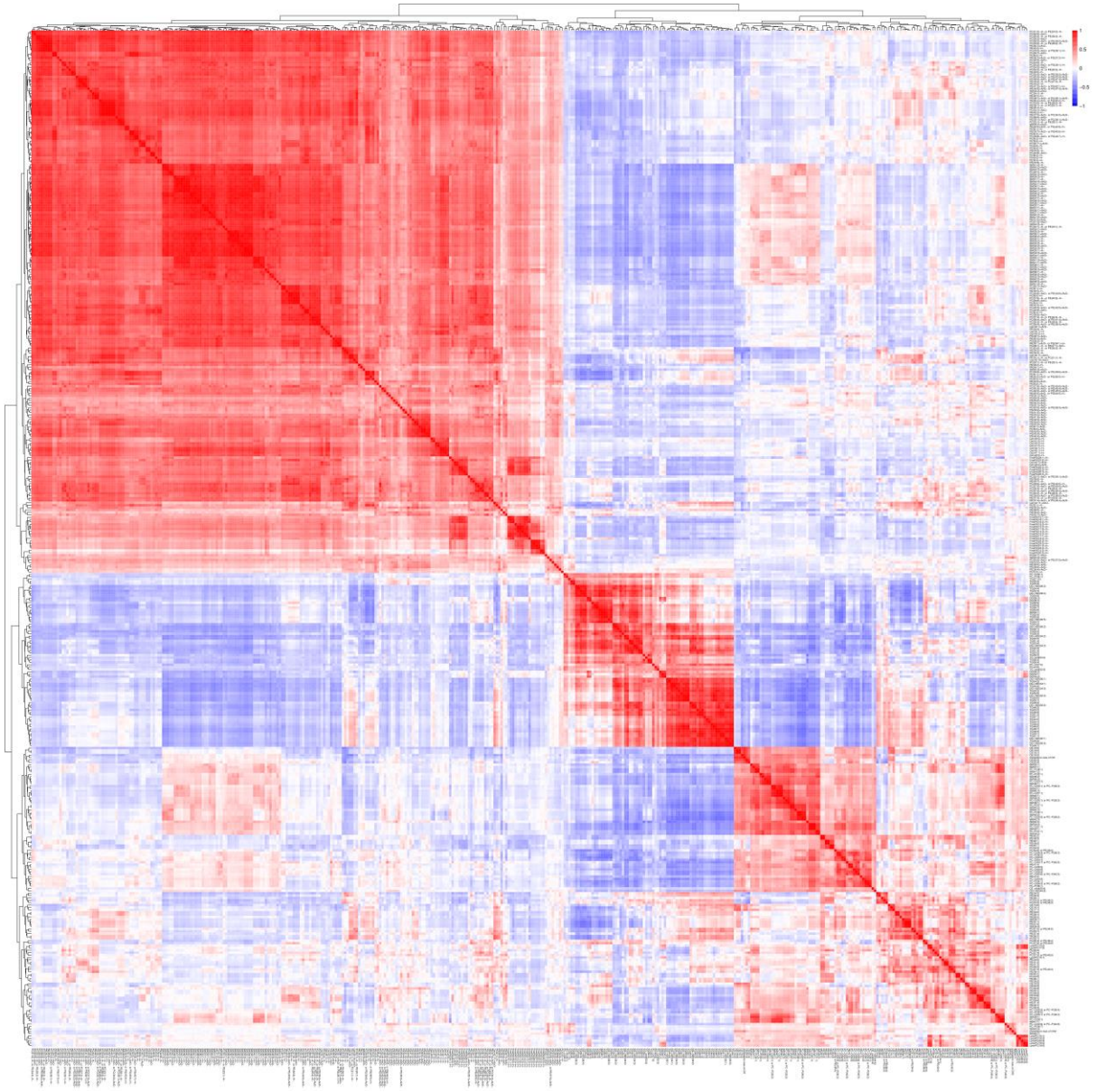


The coefficients of variation for each lipid expressed against the relative intensity for the quality control samples.

eFigure 2. Normalized relative intensity recorded for each participant by lipid mass-to-charge ratio.

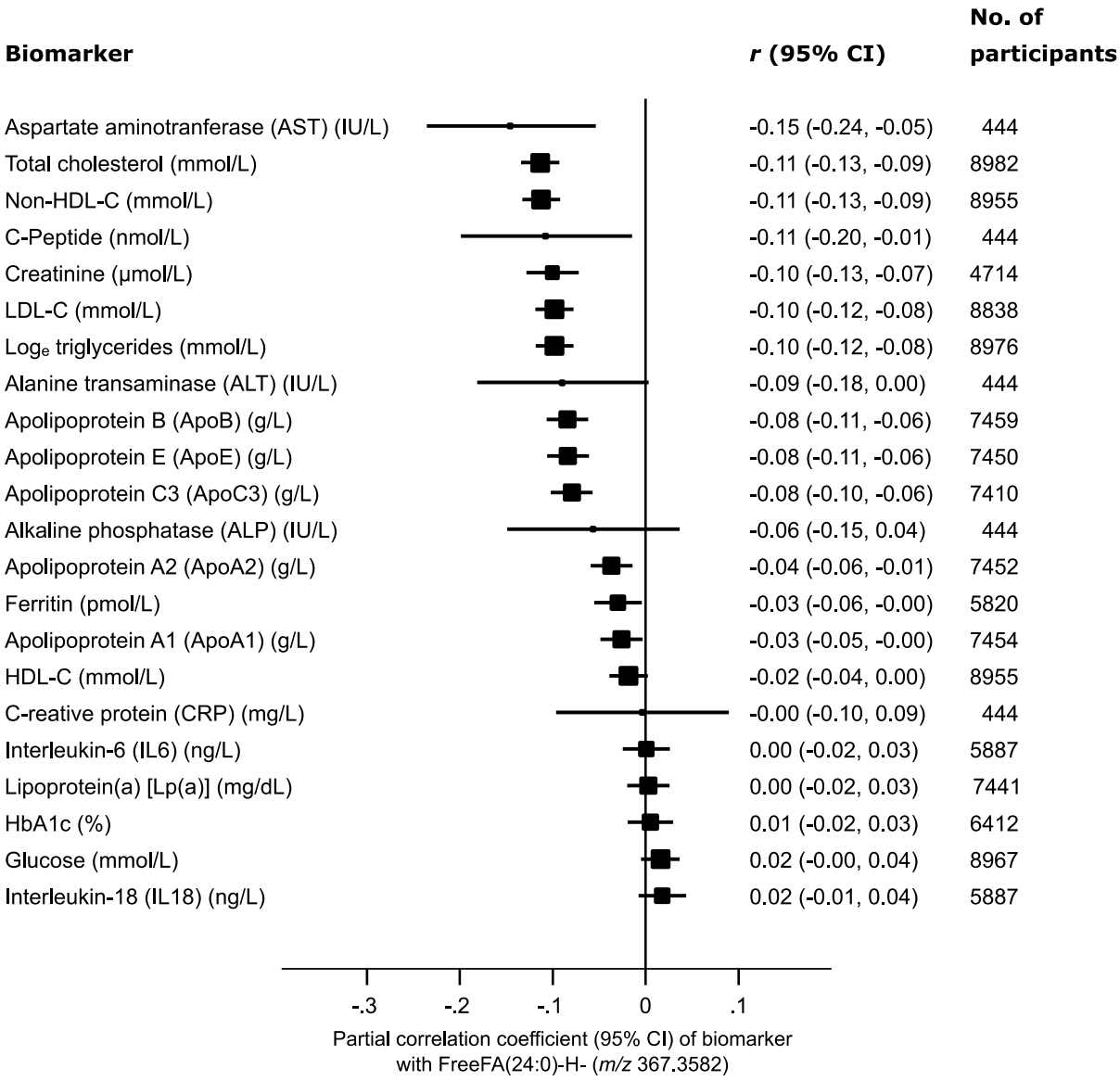


eFigure 3. Heat map of cross-correlations of lipids with hierarchical clustering

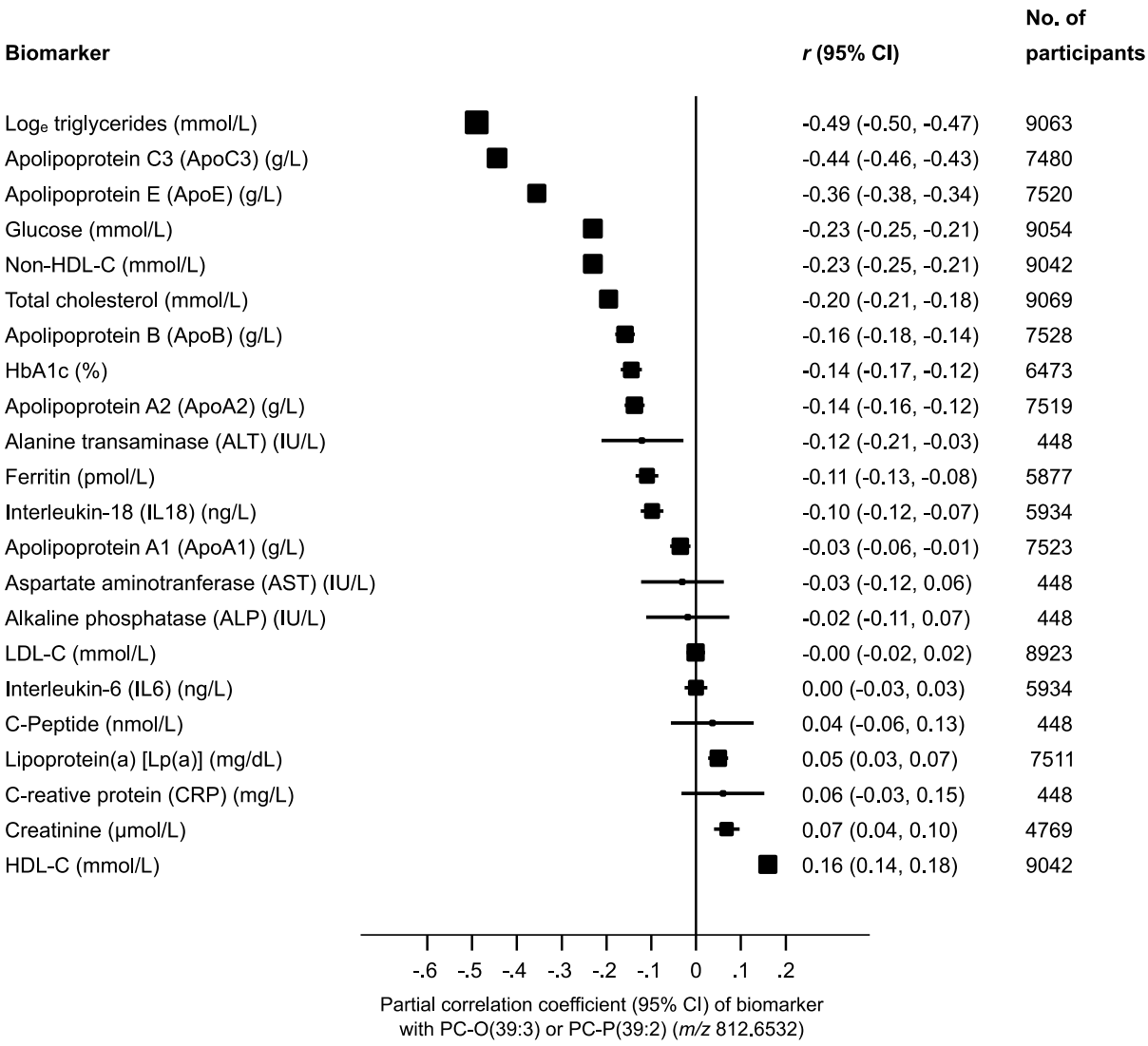


eFigure 4 Cross-correlations of circulating biomarkers with lipids that are most strongly associated with *LPL* within each overall lipid category

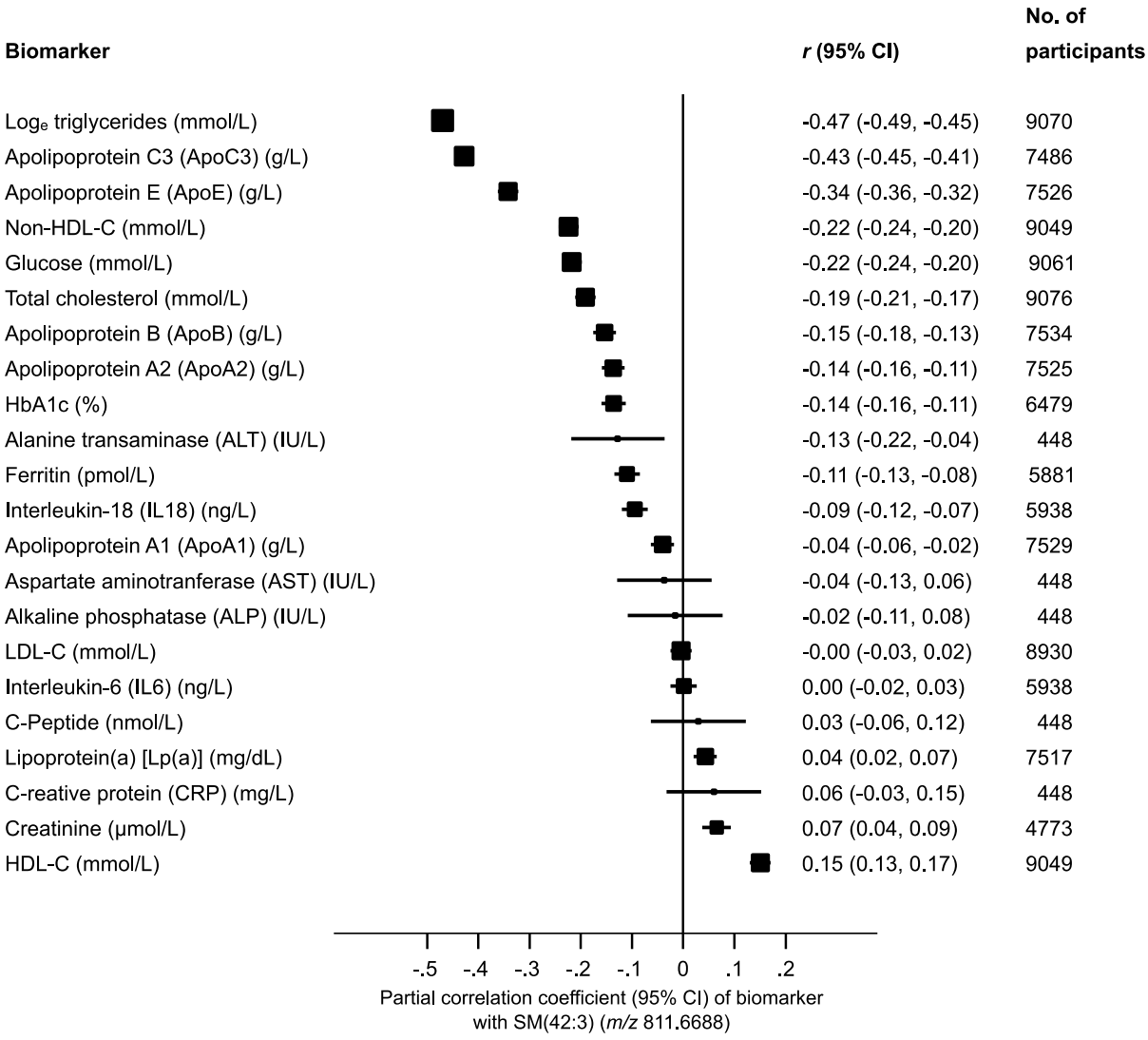
(a) Fatty acids: FreeFA(24:0)-H$^-$ (*m/z* 367.3582)



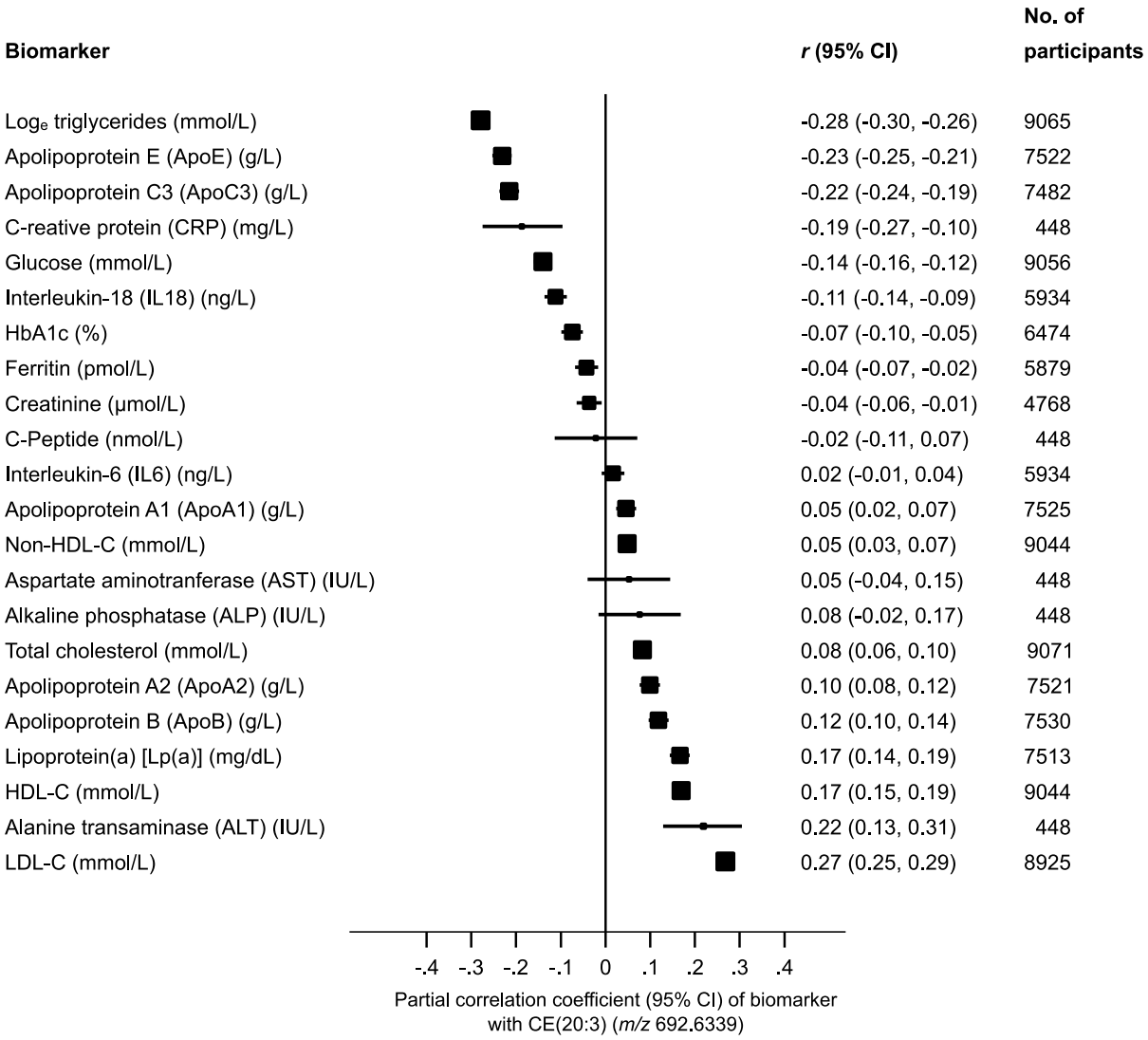
(b) Glycerophospholipids: PC-O(39:3) or PC-P(39:2) (*m/z* 812.6532)



(c) Sphingolipids: SM(42:3) (*m/z* 811.6688)

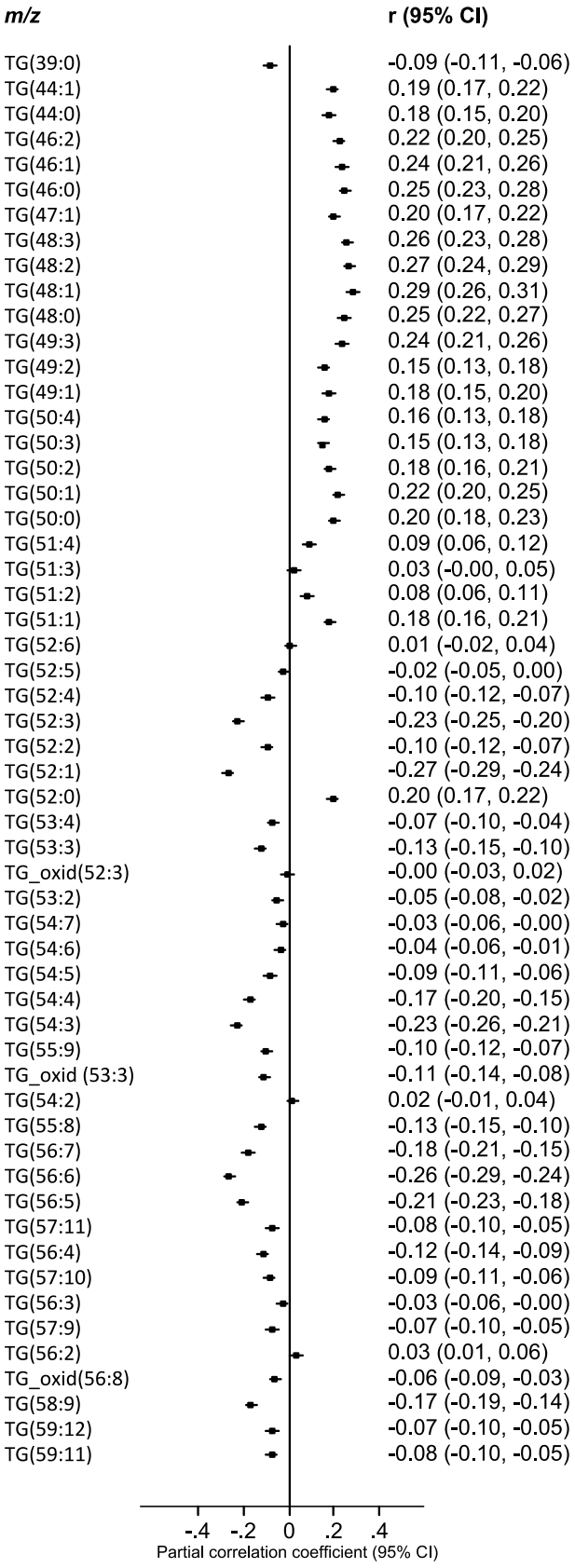


(d) Sterol lipids: CE(20:3) (*m/z* 692.6339)



All analyses were adjusted for age and sex.

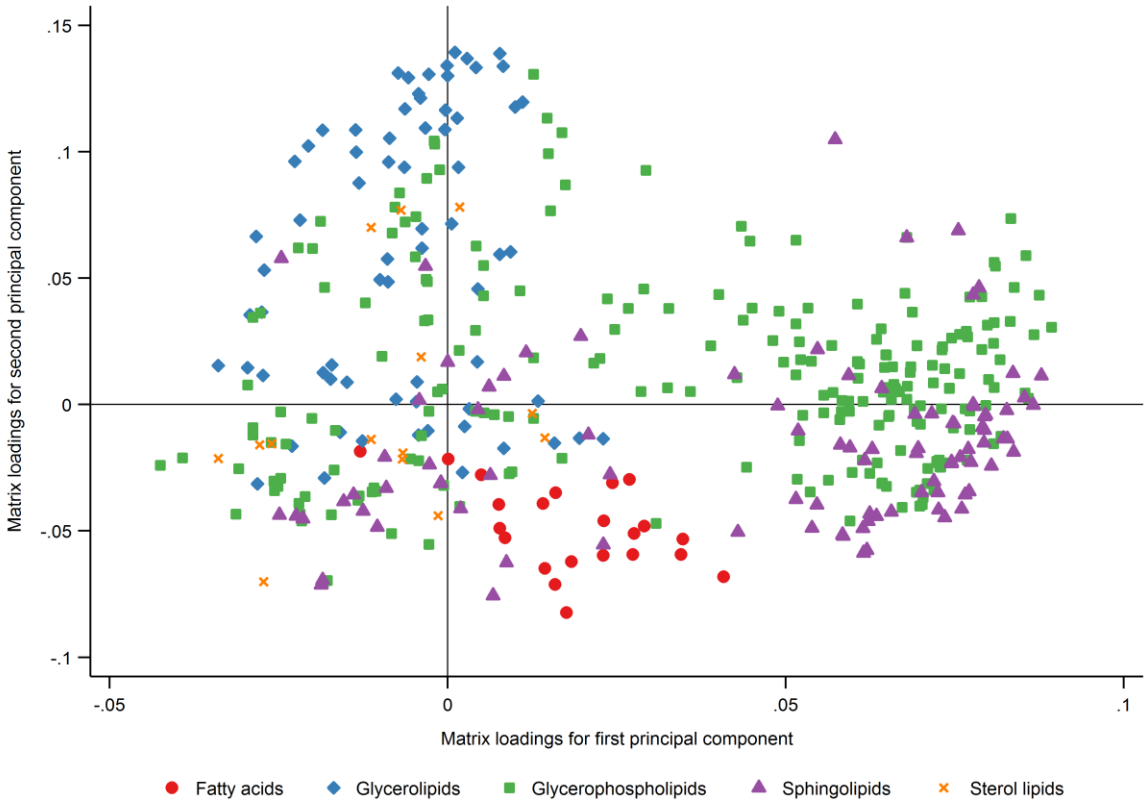
eFigure 5. Partial correlation coefficients of triglycerides measured by DIHRMS with levels of circulating VLDL



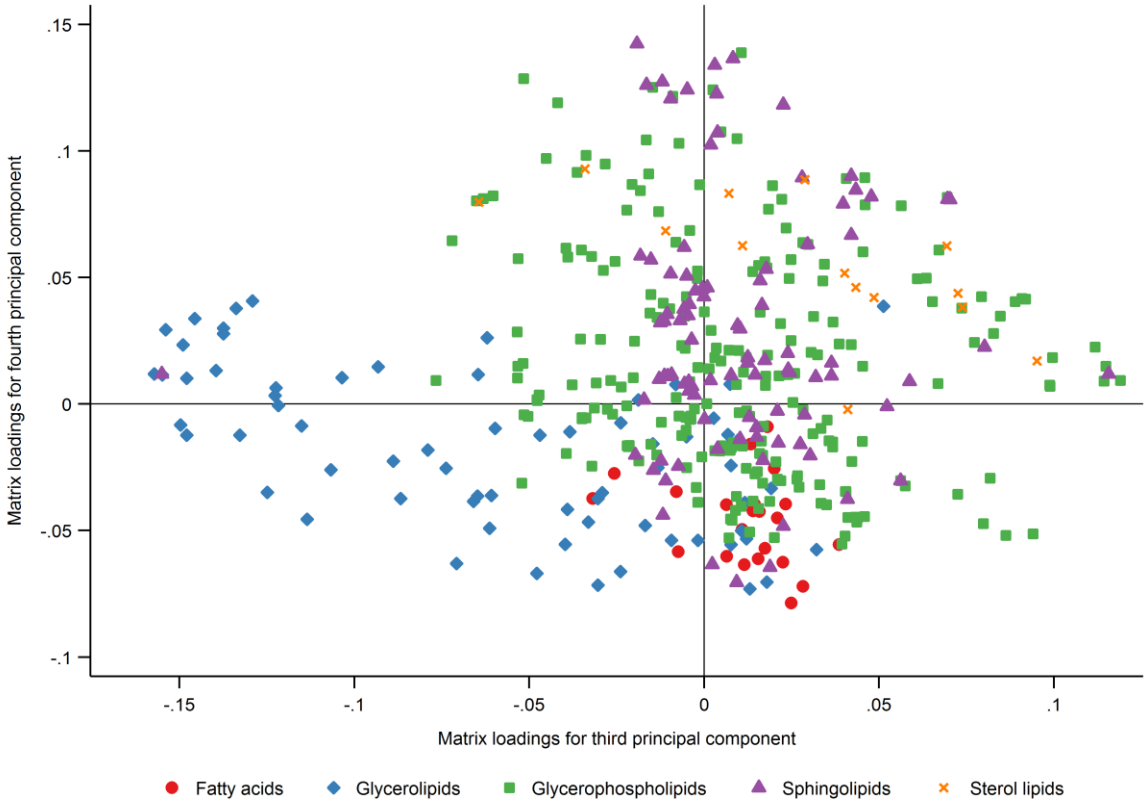
All analyses were adjusted for age and sex.

eFigure 6 Scatter plots showing matrix loadings of normalised relative intensities of lipids from principal component analysis

(a) First and second principal components



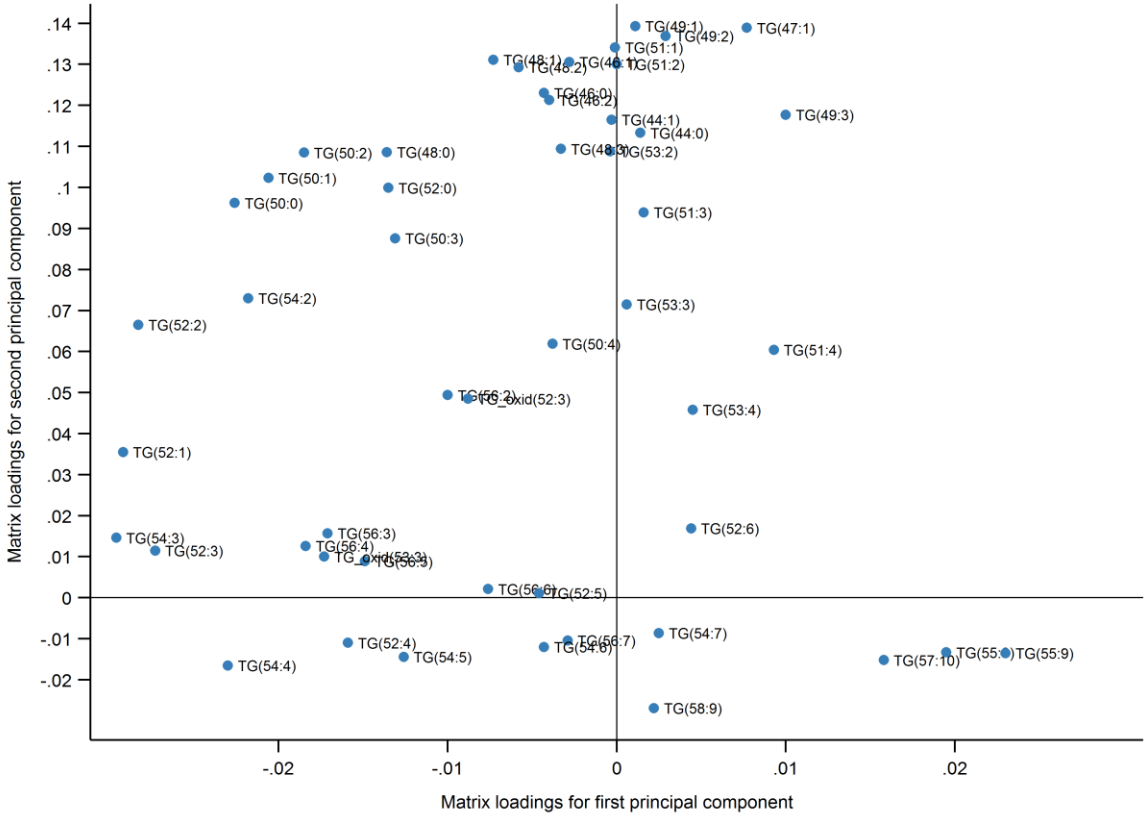
(b) Third and fourth principal components



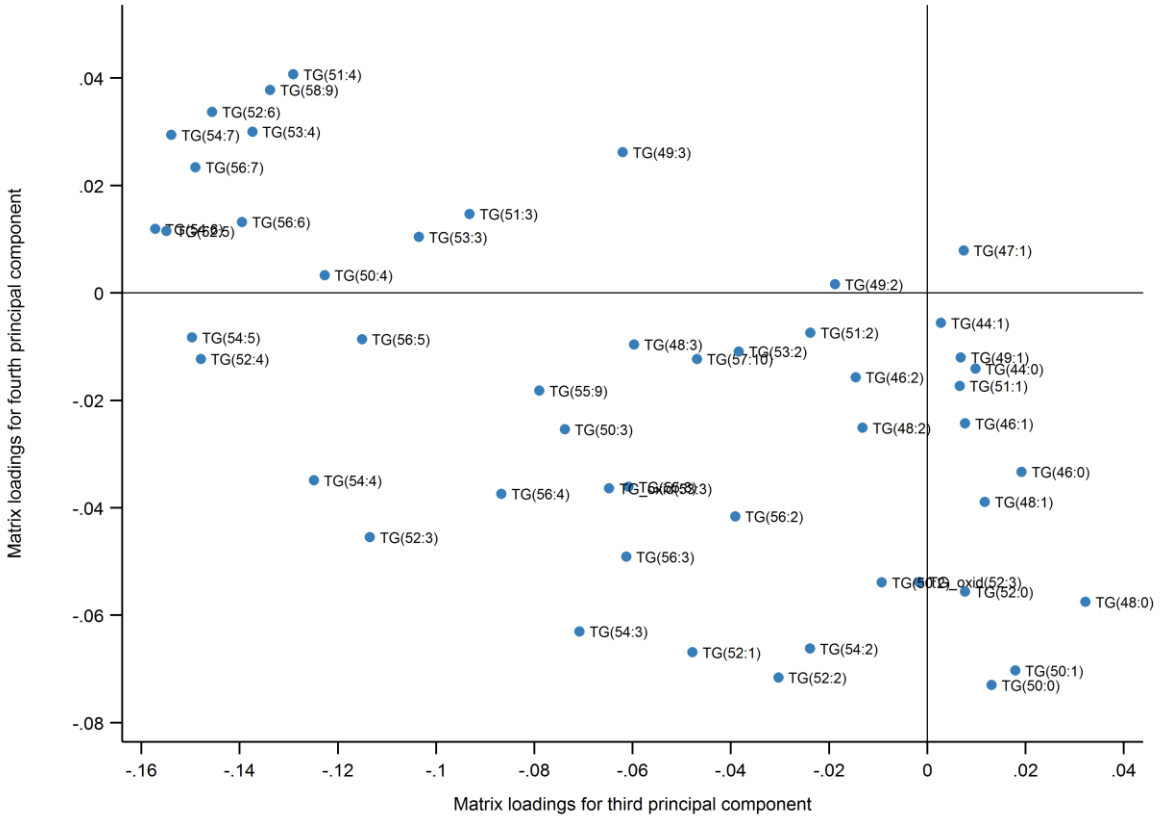
Abbreviations: FA = fatty acids; GL = glycerolipids (including diacylglycerols and triacylglycerols); **GP** = glycerophospholipids (including lysophosphatidylcholines, phosphatidylcholines, and phosphatidylethanolamines); **PC** = principal component; **SP** = sphingolipids (including sphingomyelins); **ST** = sterol lipids (including cholesteryl esters).

eFigure 7 Scatter plots showing matrix loadings of normalised relative intensities of triglycerides from principal component analysis

(a) First and second principal components

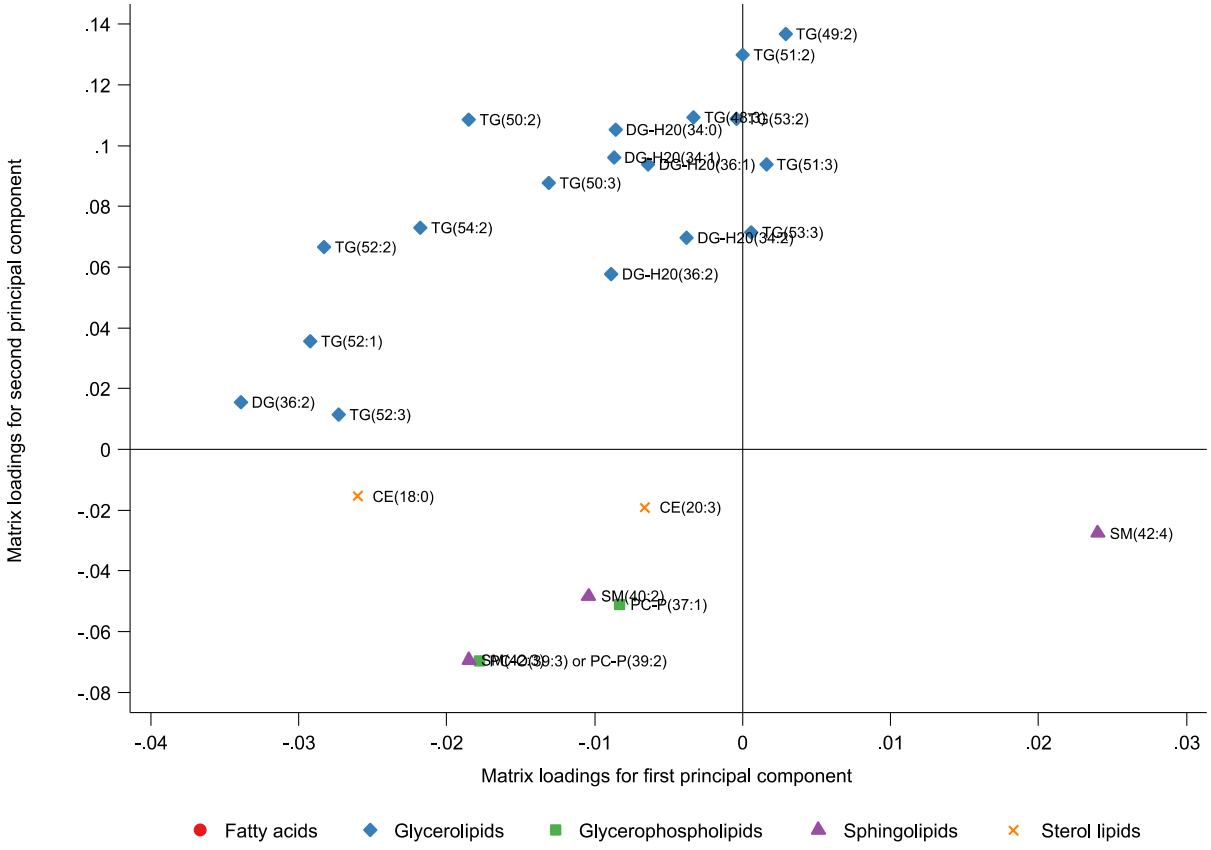


(b) Third and fourth principal components

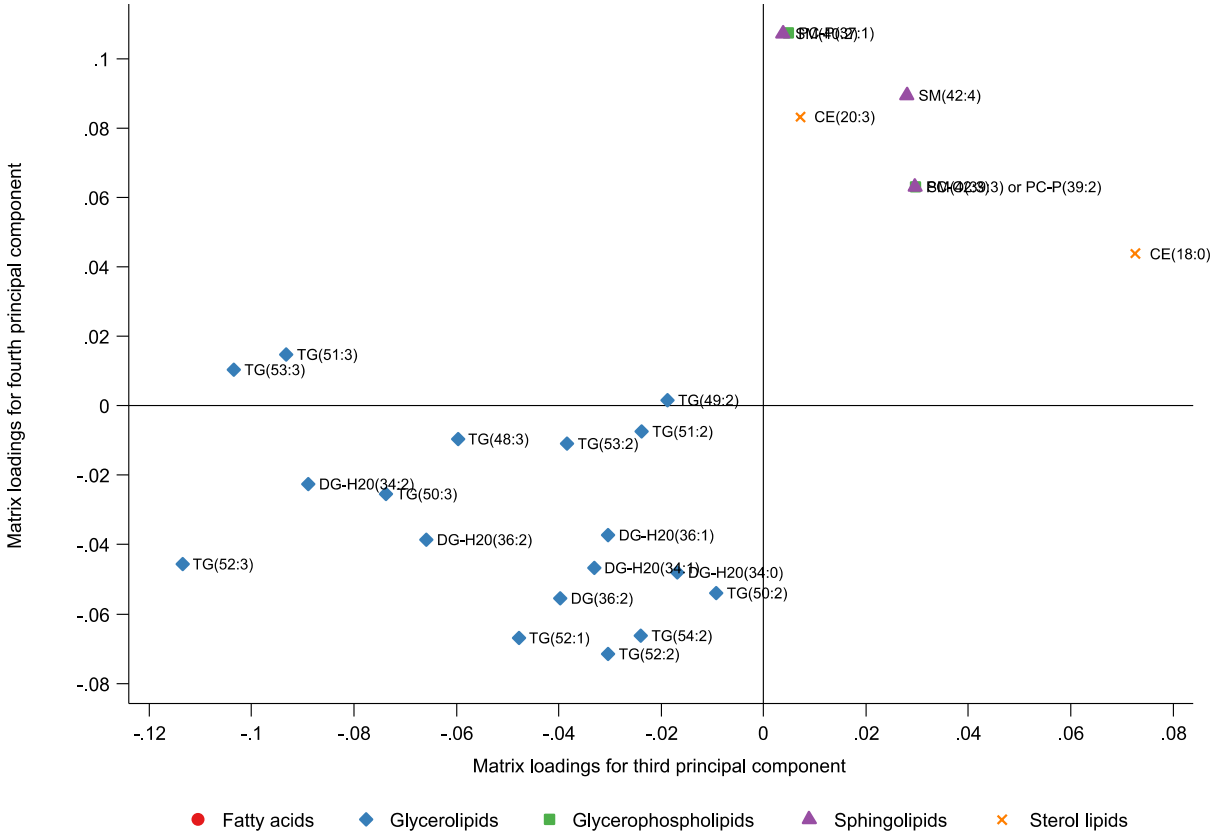


eFigure 8 Scatter plots showing matrix loadings of normalised relative intensities of lipids from principal component analysis that are significantly associated with *LPL*

(a) First and second principal components

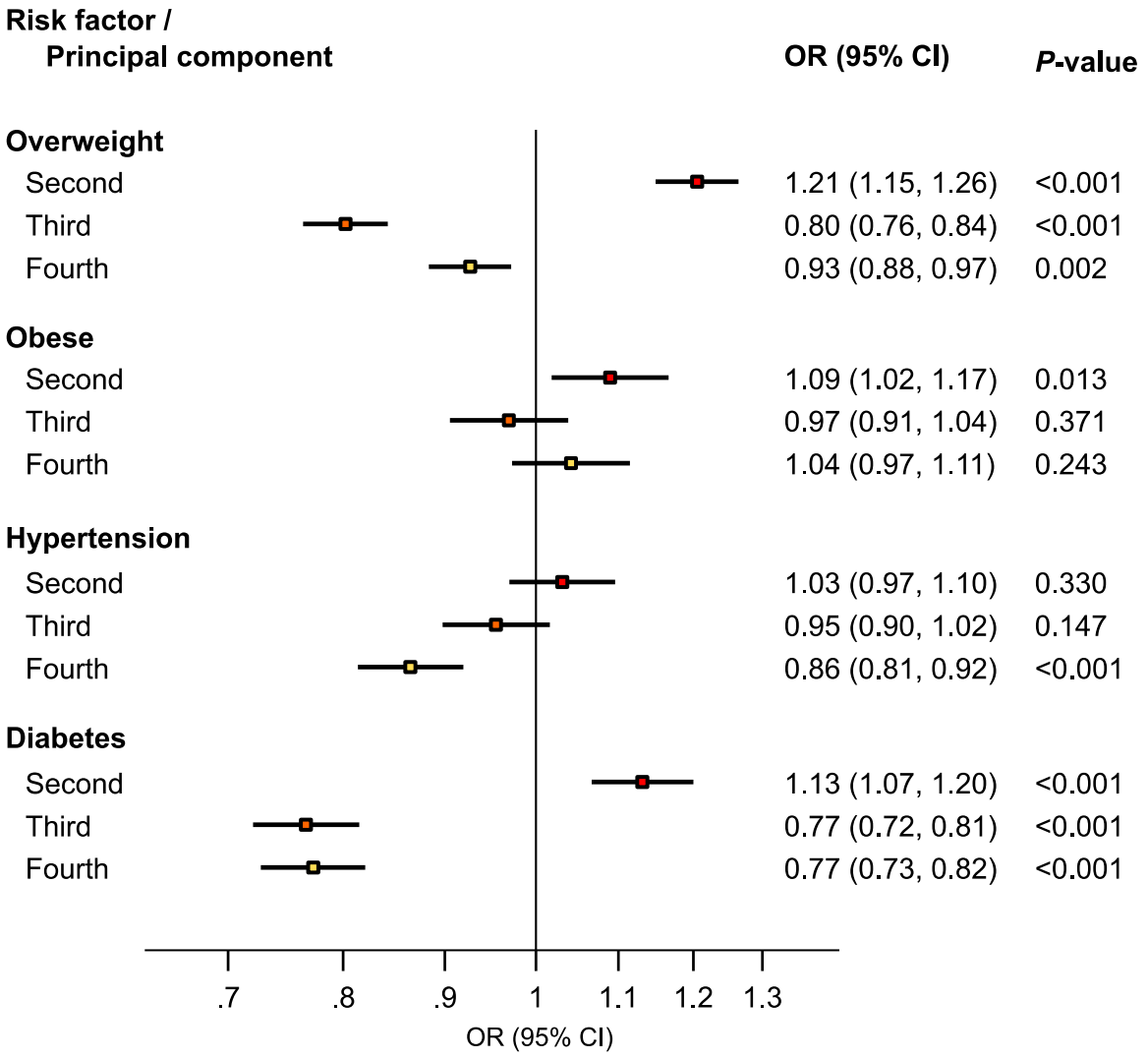


(b) Third and fourth principal components



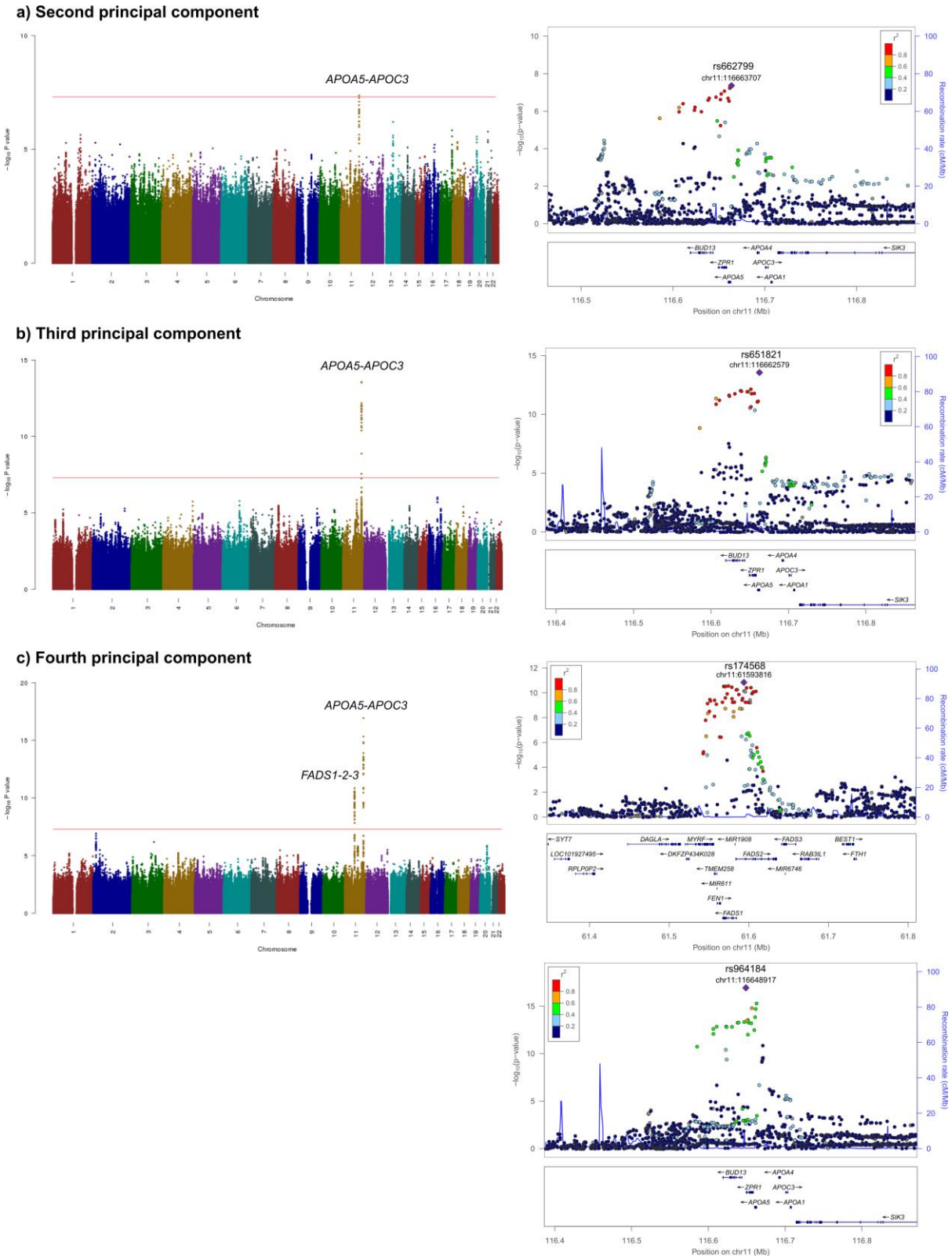
Scatter plot is shown for the subset of lipids that are significantly associated with *LPL* (rs328, chr8:19819724) at $P < 5 \times 10^{-8}$.

eFigure 9. Association of established coronary heart disease risk factors with principal components of lipid levels



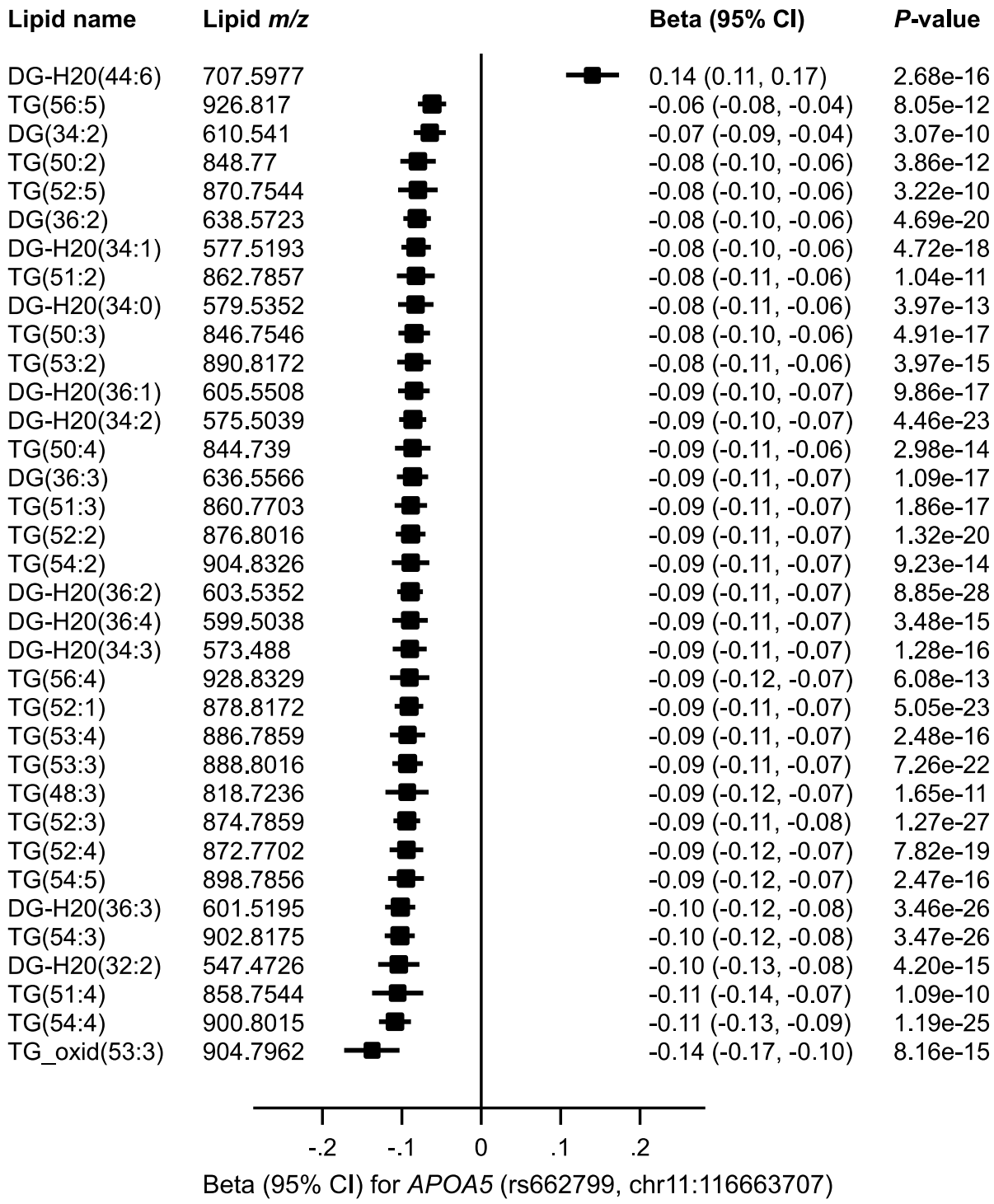
All analyses were adjusted for age and sex. Odds ratios (OR) and 95% confidence intervals (CI) for each principal component are expressed per 1-SD increase in the loading scores of the lipids that make up that component. **Definitions: Diabetes** = HbA_{1c} ≥ 6.5%; **Hypertension** = SBP ≥ 140 mm Hg or DBP ≥ 90 mm Hg; **Obese** = BMI ≥ 30 kg/m^2; **Overweight** = BMI ≥ 25 kg/m^2. **Abbreviations: BMI** = body mass index; **DBP** = diastolic blood pressure; **FPG** = fasting plasma glucose; **SBP** = systolic blood pressure.

eFigure 10. Manhattan and regional association plots of principal components of lipid levels



eFigure 11 Phenome scan of lipids that are significantly associated with *APOA5*

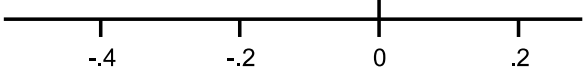
(a) Glycerolipids



Significance threshold: $P < 8.9e\text{-}10$

(b) Glycerophospholipids

Lipid name	Lipid *m/z*	Beta (95% CI)	*P*-value
PC-P(39:1)	814.6688	0.08 (0.06, 0.09)	8.29e-25
PC-O(39:3) or PC-P(39:2)	812.6532	0.08 (0.06, 0.09)	5.88e-23
PC-P(37:1)	786.6373	0.07 (0.06, 0.09)	9.77e-30
PC-P(38:1)	800.6529	0.07 (0.06, 0.09)	1.15e-23
PC-O(33:1)	732.5904	0.07 (0.06, 0.08)	1.66e-26
PC-O(31:1)	704.5591	0.07 (0.06, 0.08)	8.39e-31
PC-O(35:1) or PC-P(35:0)	760.6219	0.07 (0.06, 0.08)	3.25e-26
PC-O(37:1)	788.653	0.07 (0.06, 0.08)	5.02e-28
PC-O(39:1) or PC-P(39:0)	816.6845	0.07 (0.05, 0.08)	1.31e-21
PC-O(32:0)	720.5906	0.06 (0.05, 0.08)	1.13e-13
PC-O(38:1)	802.6686	0.06 (0.05, 0.08)	2.32e-12
PC-O(34:3)	742.5748	0.06 (0.05, 0.08)	3.48e-13
PC-O(34:1)	746.6061	0.05 (0.04, 0.07)	3.65e-12
PA(41:4)	767.559	0.05 (0.04, 0.06)	7.68e-13
PC-P(31:1)	702.5434	0.05 (0.04, 0.06)	1.81e-14
PC(32:0) or PE(35:0)	734.5699	0.05 (0.04, 0.06)	1.98e-18
PC-O(36:5)	766.5745	0.05 (0.03, 0.06)	1.33e-11
PC(45:0)-H-	914.7583	-0.24 (-0.31, -0.18)	5.09e-13

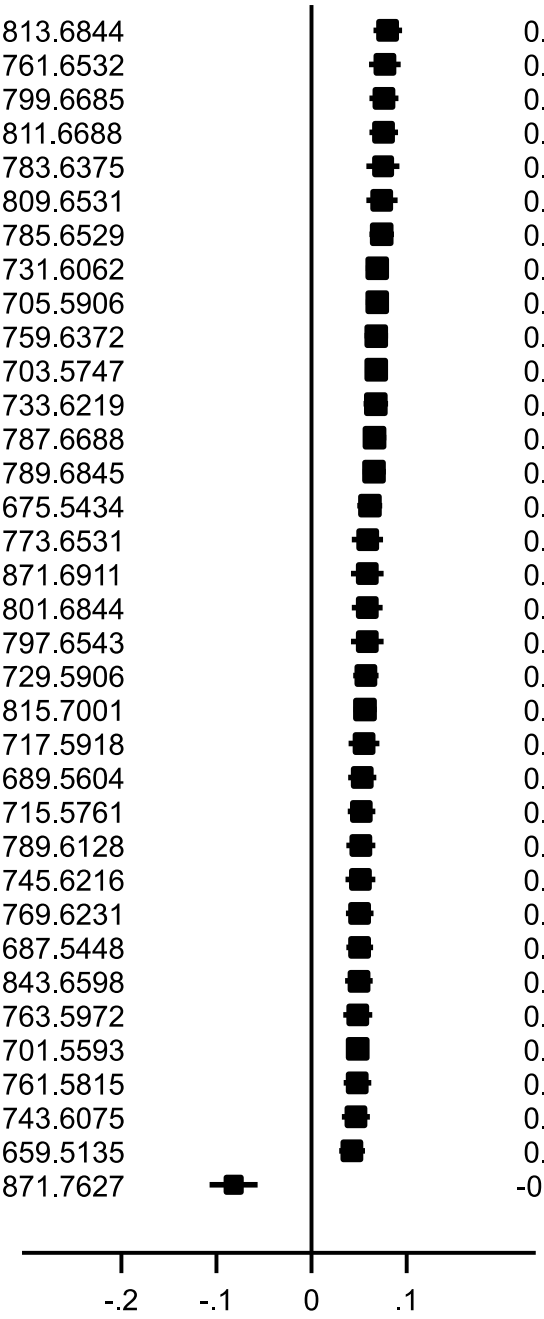


Beta (95% CI) for *APOA5* (rs662799, chr11:116663707)

Significance threshold: $P < 8.9e-10$

(c) Sphingolipids

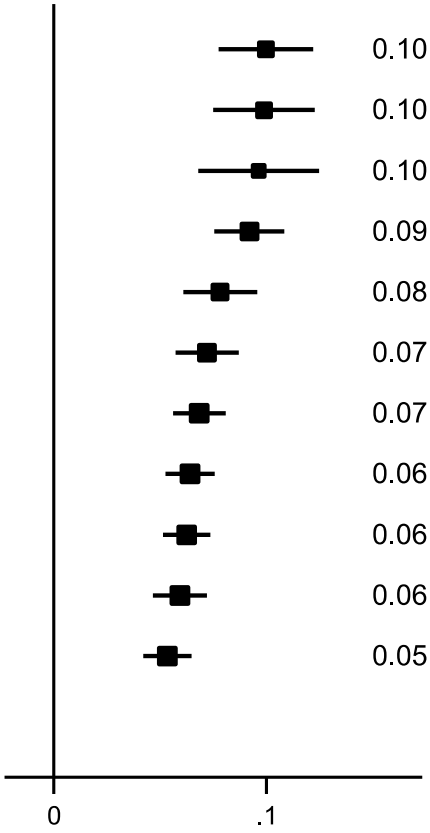
Lipid name	Lipid *m/z*	Beta (95% CI)	*P*-value
SM(42:2)	813.6844	0.08 (0.06, 0.10)	3.52e-25
SM(38:0)	761.6532	0.08 (0.06, 0.09)	5.50e-20
SM(41:2)	799.6685	0.08 (0.06, 0.09)	4.86e-23
SM(42:3)	811.6688	0.08 (0.06, 0.09)	1.17e-22
SM(40:3)	783.6375	0.08 (0.06, 0.09)	2.55e-17
SM(42:4)	809.6531	0.07 (0.06, 0.09)	8.96e-19
SM(40:2)	785.6529	0.07 (0.06, 0.09)	6.81e-30
SM(36:1)	731.6062	0.07 (0.06, 0.08)	5.11e-28
SM(34:0)	705.5906	0.07 (0.06, 0.08)	9.55e-30
SM(38:1)	759.6372	0.07 (0.06, 0.08)	3.31e-27
SM(34:1)	703.5747	0.07 (0.06, 0.08)	8.41e-30
SM(36:0)	733.6219	0.07 (0.05, 0.08)	2.09e-24
SM(40:1)	787.6688	0.07 (0.05, 0.08)	8.79e-28
SM(40:0)	789.6845	0.07 (0.05, 0.08)	2.17e-27
SM(32:1)	675.5434	0.06 (0.05, 0.07)	7.00e-20
SM(39:1)	773.6531	0.06 (0.04, 0.08)	3.13e-12
SM(42:2)+AcO-	871.6911	0.06 (0.04, 0.08)	1.90e-11
SM(41:1)	801.6844	0.06 (0.04, 0.07)	1.70e-12
SM(41:2)-H-	797.6543	0.06 (0.04, 0.08)	2.24e-11
SM(36:2)	729.5906	0.06 (0.04, 0.07)	4.75e-17
SM(42:1)	815.7001	0.06 (0.04, 0.07)	2.27e-19
SM(35:0)-H-	717.5918	0.06 (0.04, 0.07)	3.68e-11
SM(33:0)-H-	689.5604	0.05 (0.04, 0.07)	1.93e-12
SM(35:1)-H-	715.5761	0.05 (0.04, 0.07)	2.65e-12
SM(36:1)+AcO-	789.6128	0.05 (0.04, 0.07)	2.06e-11
SM(37:1)	745.6216	0.05 (0.04, 0.07)	6.48e-11
SM(39:2)-H-	769.6231	0.05 (0.04, 0.07)	1.24e-11
SM(33:1)-H-	687.5448	0.05 (0.04, 0.06)	2.89e-12
SM(40:2)+AcO-	843.6598	0.05 (0.04, 0.06)	1.84e-11
SM(34:0)+AcO-	763.5972	0.05 (0.03, 0.06)	4.67e-10
SM(34:2)	701.5593	0.05 (0.04, 0.06)	2.15e-17
SM(34:1)+AcO-	761.5815	0.05 (0.03, 0.06)	1.07e-10
SM(37:1)-H-	743.6075	0.05 (0.03, 0.06)	8.36e-10
SM(31:1)-H-	659.5135	0.04 (0.03, 0.06)	7.25e-10
SM(46:1)	871.7627	-0.08 (-0.11, -0.06)	2.14e-10



Significance threshold: $P < 8.9e\text{-}10$

(d) Sterol lipids

Lipid name	Lipid *m/z*	Beta (95% CI)	*P*-value
CE(16:1)	640.6024	0.10 (0.08, 0.12)	2.19e-18
CE(17:1)	654.618	0.10 (0.07, 0.12)	4.18e-16
CE(20:5)	688.6026	0.10 (0.07, 0.12)	2.65e-11
CE(18:3)	664.6026	0.09 (0.08, 0.11)	7.34e-28
CE(20:4)	690.6183	0.08 (0.06, 0.10)	1.58e-18
CE(20:3)	692.6339	0.07 (0.06, 0.09)	3.97e-21
CE(18:0)	670.6496	0.07 (0.06, 0.08)	9.01e-28
CE(18:1)	668.6339	0.06 (0.05, 0.08)	1.37e-27
cholesterol-loss of OH	369.3514	0.06 (0.05, 0.07)	4.55e-28
CE(18:2)	666.6183	0.06 (0.05, 0.07)	1.25e-19
CE(16:0)	642.6183	0.05 (0.04, 0.06)	4.07e-20



Beta (95% CI) for *APOA5* (rs662799, chr11:116663707)

Significance threshold: $P < 8.9e\text{-}10$