Supplementary Material

Large-scale plasma lipidomic profiling identifies lipids that predict cardiovascular events in secondary prevention

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Lipid analysis

Lipid species were extracted from plasma samples as described previously (1). Briefly, plasma (10 µL) was aliquoted into a 1.5 mL eppendorf tube using a positive displacement pipette and 100 µL of 1-butanol/methanol (1:1, v/v), 5 mM ammonium formate containing internal standards (Supplementary Table 1) was added, also using positive displacement pipettes. The mixture was vortexed for 10 seconds, sonicated for 60 minutes in a sonic water bath (18°C-24°C) and then centrifuged (16,000xg, 10 min, 20°C). The supernatant was transferred into a 0.2 mL glass insert with teflon insert caps for lipidomic analysis.

The lipidomic methodology used for this study was an advance upon our earlier targeted methodology developed on an Agilent 1200 liquid chromatography system combined with an Applied Biosystems API 4000 Q/TRAP mass spectrometer (2). In this study lipidomic analysis was performed by liquid chromatography electrospray ionisation tandem mass spectrometry on an Agilent 1290 liquid chromatography system combined with an Agilent 6490 triple quadrupole mass spectrometer, utilizing Mass Hunter software. Liquid chromatography was performed on a Zorbax Eclipse Plus 1.8 µm C18, 50 × 2.1 mm column (Agilent Technologies). Solvents A and B consisted of tetrahydrofuran:methanol:water in the ratio (20:20:60) and (75:20:5) respectively, both containing 10 mM ammonium formate. Columns were heated to 50°C and the auto-sampler regulated to 25°C. Lipid species (1 µL injection) were separated under gradient conditions at a flow rate of 400 µL/min. The gradient was as follows; 0% solvent B to 40% solvent B over 2.0 min, 40% solvent B to 100% solvent B over 6.5 min, 0.5 min at 100% solvent B, a return to 0% solvent B over 0.5 min then 0.5 min at 0% solvent B prior to the next injection (total run time of 10 min).

The mass spectrometer was operated in dynamic/scheduled multiple reaction monitoring (dMRM) mode. There were 345 unique lipid species measured together with 16 stable isotope or non-physiological lipid standards (Supplementary Table 1 and 3). Mass spectrometer voltages used for the acquisition of data were; fragmentor voltage, 380 V and cell accelerator voltage, 5 V. The collision

energy voltage was set individually for each lipid class and subclass and is listed in Supplementary Table 1. Acquisition windows were set to between 0.7 and 1.76 min depending on the chromatographic properties of the lipid. Further, there were several sets of isobaric lipids which shared the same nominal parent ion mass and also give rise to the same product ions. Specifically, for isobaric species of phosphatidylcholine, alkylphosphatidylcholine and alkenylphosphatidylcholine the parent and product ions (m/z 184) were the same. As a result a single MRM transition was used to measure the corresponding species within each subclass, using an increased MRM window time (21 combinations). Additionally there was one further occurrence of isobaric phosphatidylethanolamine and alkylphosphatidylethanolamine lipid species, representing the neutral loss of 141 Da, which were similarly combined into a single dMRM transition (3).

While most lipid classes and subclasses have similar response factors for lipid species within the class, some classes show greater variation in response factors between species. Consequently, correction factors were applied for some lipid classes as we have described earlier (1) but now adjusted for the Agilent mass spectrometer.

Diacyl- and triacylglycerol: Fragmentation of the ammoniated adducts of diacyl- and triacylglycerol leads to the loss of ammonia and a fatty acid. In this context it is important to recognize that for species which contain more than one of the same fatty acid, the loss of that fatty acid will result in an enhanced signal, as it is the end product from two competing pathways. Consequently, where we used an MRM transition that corresponded to the loss of a fatty acid that was present more than once, we divided by the number of times that fatty acid was present. While we recognize that the response factor for different species of triacylglycerol varied substantially, the lack of suitable standards precluded the determination of suitable response factors for each triacylglycerol species.

Cholesteryl ester: Response factors were determined with seven commercially available species and used to create a formula to extrapolate for all cholesteryl ester chain lengths and double bonds. Saturated species were characterized by the following relationship: $y = 0.1486x - 1.5917$, where y is the response factor relative to the CE 18:0 d 6 internal standard and x is the carbon chain length. For monounsaturated species, the response factor was multiplied by 1.84 and for polyunsaturated species by 6.0.

Phosphatidylinositol: A single response factor was calculated for all phosphatidylinositol species to account for the use of the phosphatidylethanolamine (PE(17:0/17:0)) as the internal standard for this lipid class. A nine point standard curve was created using commercially available phosphatidylinositol (PI(32:0)) and subsequently spiked into solvent containing a fixed concentration of PE(17:0/17:0). The standard curve resulted in a linear response and indicated a response factor of 1.44 for phosphatidylinositol species relative to phosphatidylethanolamine standard. Other lipid species were not corrected.

Quality control samples

Two types of quality control samples were utilized in this study. Plasma from six healthy volunteers was pooled and split into multiple aliquots. We refer to these samples as plasma quality control (PQC) samples. These samples are then subjected to extraction and LC-MS analysis alongside samples from the study to provide a measure of analytical variability across the study as a whole.

Additionally we utilized identical lipid extracts, which were prepared by pooling the lipid extracts from multiple PQC samples using this mixture to prepare multiple aliquots which were referred to as technical quality control (TQC) samples. Analysis of these samples captures only the variation

associated with the LC-MS performance. Within the analytical process every twenty plasma samples a PQC and TQC were included.

Pre-processing of data

In this study, samples were run in multiple batches. An extraction batch consisted 448 plasma samples, 24 PQC, 24 TQC and 12 blank samples (resulting in 27 batches). Two batches were run consecutively between cleaning of the mass spectrometer. A median centering approach was used for correction of the batch effect. The median PQC concentration of each lipid for each batch was used as a reference point to align the samples with the entire cohort. The alignment was performed by calculating a correction factor to adjust the concentration of each PQC lipid in each batch to the median value for all batches.

Where lipid values were below the limit of quantification the value was assigned as half the lowest quantified value. No lipid species has more than 1% of samples below the limit of quantification.

Multiple imputation by chained equations (MICE) (4) was used to impute missing clinical covariates (BMI, n=1; FBG, n=13 and WBC, n=1) using all 22 clinical covariates, prior to statistical analysis.

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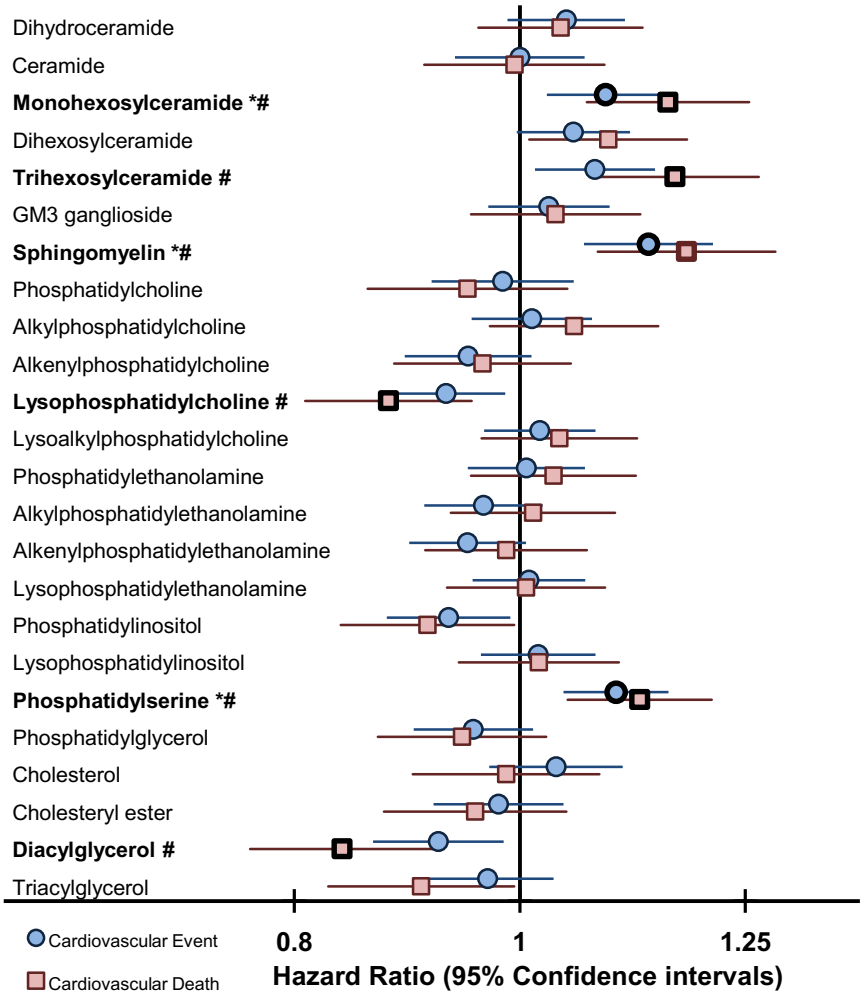
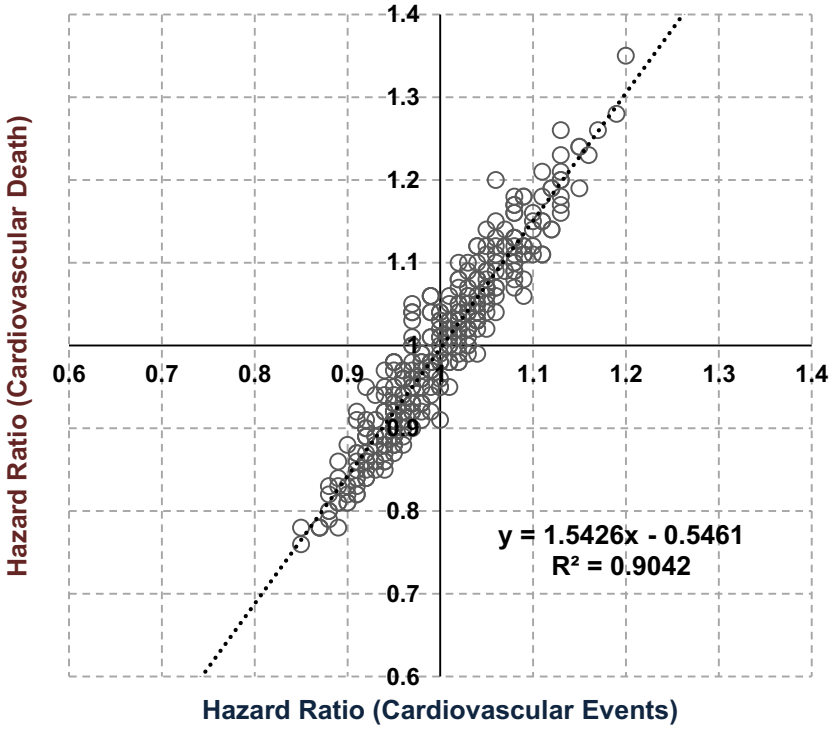
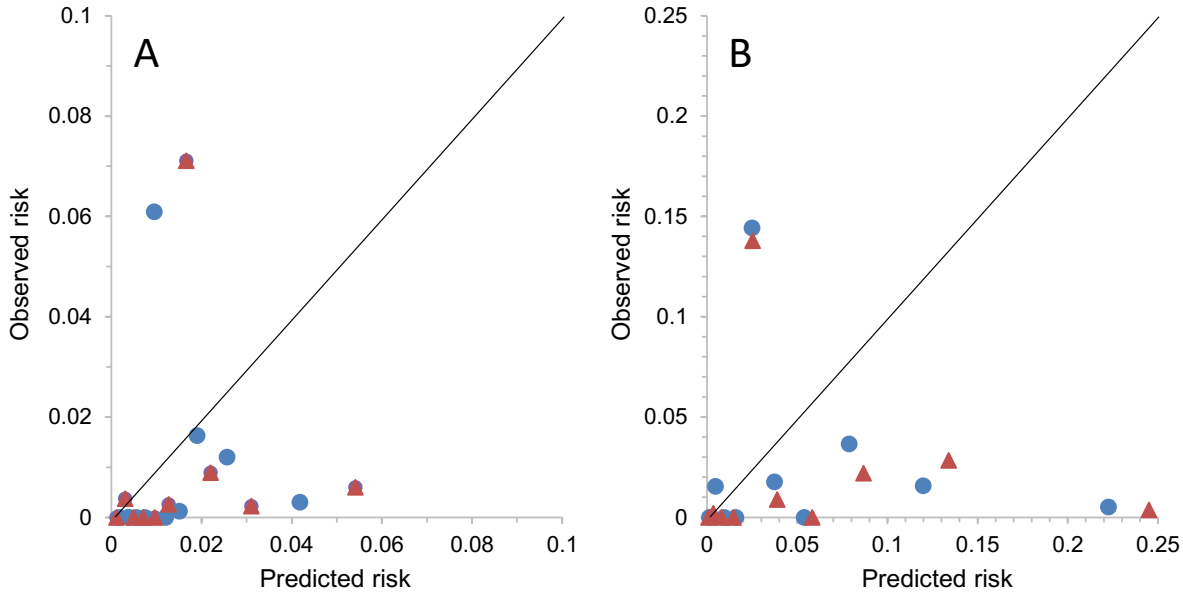


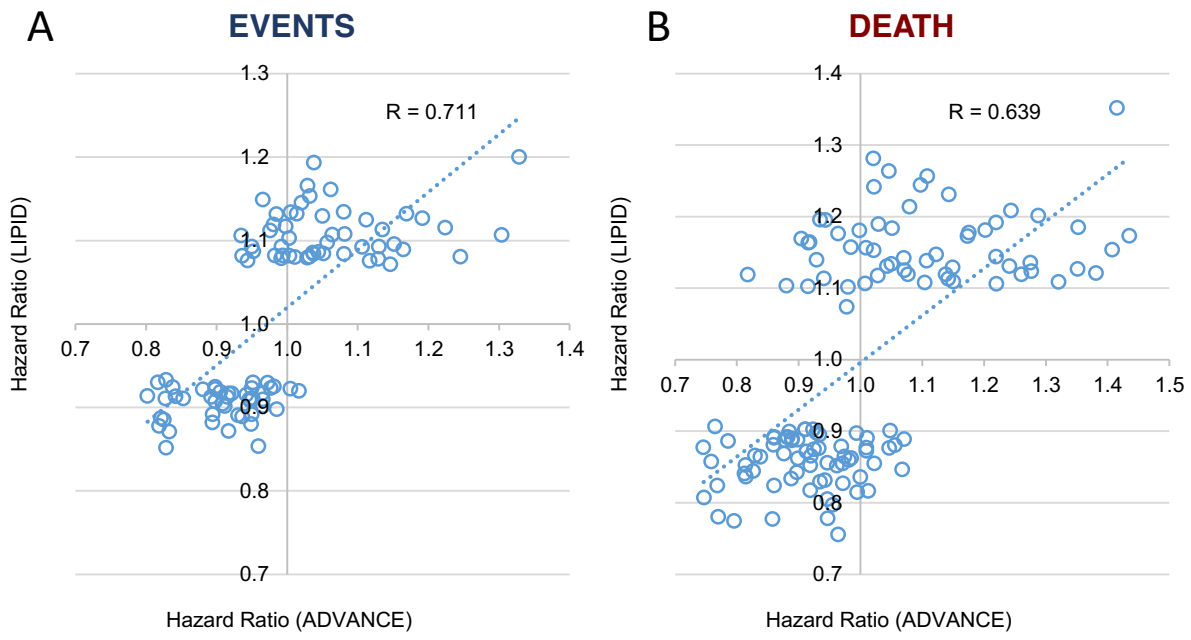
Supplementary Figure 1. Plasma lipid classes associated with future cardiovascular events and cardiovascular death. Cox regression models of each lipid class against cardiovascular events (closed circle) and cardiovascular death (open square) were created adjusting for 11 covariates (total cholesterol, HDL-C, current smoking, nature of prior acute coronary syndrome, revascularization, diabetes history, stroke history, history of hypertension and randomised treatment allocation). Hazard ratios per unit standard deviation, and 95% confidence intervals are shown. * significant association with cardiovascular events, # significant association with cardiovascular death.



Supplementary Figure 2: Correlation between hazard ratios for cardiovascular events and hazard ratios for cardiovascular death. A Cox regression analysis was performed to identify lipids associated with cardiovascular events and cardiovascular death in the LIPID subcohort. The models were adjusted for 11 covariates (age, gender, total cholesterol, HDL-C, current smoking, nature of prior acute coronary syndrome, revascularization, diabetes history, stroke history, history of hypertension and randomised treatment allocation).



Supplementary Figure 3: Calibration plots of risk prediction models. Calibration Plots for the models predicting cardiovascular events (panel A) and cardiovascular death (panel B) on the LIPID cohort. Blue circles represent the base models and red triangles denote the models with both base covariates and lipid variables.



Supplementary Figure 4: Hazard ratios of significant lipid species in the LIPID subcohort and ADVANCE case-cohort. Cox regression and weighted Cox regression analyses were performed to identify lipids associated with cardiovascular events and cardiovascular death in the LIPID subcohort and ADVANCE case-cohort. Hazard ratios of lipid species associated with cardiovascular events (panel A) and cardiovascular death (panel B) in the LIPID subcohort were plotted against the corresponding hazard ratios in the ADVANCE case-cohort.

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