SUPPLEMENTARY MATERIAL

Changes in plasma lipid species predicts pravastatin treatment efficacy in secondary prevention

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Study populations

In the LIPID study design [1], patients who experienced heart failure were excluded. Before the actual trial, a single-blind placebo run-in phase was conducted for 8 weeks. The study contained 9014 patients (7498 men and 1516 women). Detailed lipidomic profiling was conducted on 4,991 participants (49.7% on pravastatin treatment) using both baseline and one-year follow up

plasma samples (Supplementary Figure 1). Of these, 91 experienced a cardiovascular event within the first year. Out of the remaining 4,900 patients 944 experienced cardiovascular events (non-fatal MI, non-fatal stroke and cardiovascular death) in the subsequent follow up period (1 year to a maximum 10 years, average of 7.4 years), with 498 cardiovascular deaths. Major cardiovascular events and deaths were adjudicated by an expert committee blinded to treatment allocation.

The LIPID study was approved by the Ethics Committees of the participating institutes, all participants provided written informed consent. The subsequent analysis of archived samples was approved by the Alfred Hospital Ethics Committee.

Lipid Nomenclature

We have followed the guidelines established by the Lipid Maps Consortium and added to by Liebisch et al. (Fahy et al., 2005; Fahy et al., 2009; Liebisch et al., 2013). Glycerophospholipids typically contain two fatty acid chains and, in the absence of detailed characterization, are expressed as the sum composition of carbon atoms and double bonds (i.e. PC(38:4). However, where the acyl chains have been determined but the position is unknown, this is reflected by an underscore between the acyl chains (i.e. PC(38:6) is changed to PC(18:0_20:4)). Where the position of the acyl chains is knows the acyl chains are separated by a / with the sn1 and sn2 acyl chains in order (i.e. PC(18:0_20:4) is changed to PC(18:0_20:4)). This is also extended into other lipid classes and subclasses.

Lipid extraction and profiling

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 at 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 analysis was performed on an Agilent 6490 QQQ mass spectrometer with an Agilent 1290 series HPLC (Agilent Technologies, Santa Clara, California, United States). The mass spectrometer was operated in positive ion mode with dynamic/scheduled multiple reaction monitoring (dMRM). There were 345 unique lipid species measured together with 16 stable isotope or non-physiological lipid standards (Supplementary Table 1). 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 chromatographically separated 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. While most lipid classes and subclasses have similar response factors for lipid species within the class, some classes show greater variation in intraclass response factors. Consequently, correction factors were applied for some lipid classes as we have described earlier [2], with minor adjustments for the Agilent mass spectrometer.

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, prepared by pooling lipid extracts from multiple PQC samples, to prepare multiple aliquots which were referred to as technical quality control (TQC) samples. Analysis of these samples captures only the variation associated with LC-MS performance. Within the analytical process, a PQC and TQC sample was included every twenty plasma samples.

Pre-processing of data

In this study, samples were run in multiple batches. An extraction batch consisted of 448 plasma samples, 24 PQC, 24 TQC and 12 blank samples (resulting in 27 batches). Batches were run consecutively, with cleaning of the mass spectrometer following every second batch. A median centering approach was used for correction of the batch effect [3]. 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.

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Supplementary Figure 1: Consort diagram for patient flow. From the 9014 participants that were randomized to receive pravastatin treatment, baseline samples were available for 5991 participants and 1 year follow-up samples were available for 5782 participants. Participants for whom both baseline and follow-up samples were available (n=4991) were used in this study for subsequent analyses. Of these 4991 participants, 91 experienced a CVE within the first year and were removed from the CVE analysis (resulting in n=4900, with 944 CVE). All 4991 samples were used in the analysis of CVD death (498 CVD deaths) as there were no CVD deaths within the first year in this subcohort.

Supplementary Figure 2: Schematic diagram of mediation analysis procedure. Causal mediation analysis is conditional upon **(A)** an association between the predictor (statin treatment) and outcome (cardiovascular events) and **(B)** an association between the predictor (statin

treatment) and the mediator (deltaLR / deltaLDL-C). **(C)** Mediation analysis then determines the proportion of risk in the Outcome Model explained by a direct effect of statins on cardiovascular outcomes, the Average Direct Effect (ADE), and the proportion that is mediated by DeltaLR / delta-LDL-C, the Average Causally Mediated Effect (ACME). An interaction test for differences in ACME under different treatment status was performed. In this analysis the following definitions apply: The Total Effect is defined as the expected change in the outcome (cardiovascular events) as the predictor (statin treatment) changes from placebo to treatment, while the mediator (deltaLR / DeltaLDL-C) is allowed to track the change in the predictor. The Average Direct Effect is defined as the expected change in the outcome (cardiovascular events) as the predictor (statin treatment) changes from placebo to treatment while maintaining the mediator (deltaLR / DeltaLDL-C) at the placebo levels. The Average Causally Mediated Effect is defined as the expected change in the outcome (cardiovascular events) when the predictor (statin treatment) is maintained as treatment and the mediator (deltaLR / DeltaLDL-C) changes from the placebo values to the treated values. The Total Effect is then the sum of the Average Direct Effect and Average Causally Mediated Effect.

Supplementary Figure 3: Association of change in lipid concentration with future cardiovascular events within the treatment group. Models were adjusted for age, sex, body mass index, change in cholesterol, change in HDL-C, change in triglycerides and baseline level of a given lipid species. The hazard ratio denotes the risk per interquartile range. P-values were corrected for multiple comparisons using the Benjamini-Hochberg method. Lipid species with uncorrected P-value<0.01 are presented.

Supplementary Figure 4: Change in the lipid ratio and LDL-C in quartiles of the treatment group stratified on the change in the lipid ratio. The treatment group was stratified into quartiles using the change in the lipid ratio PI(36:2)/PC(38:4). Panel A shows the median change of LDL-C in each quartile with the interquartile range shown by the whiskers. The median change and interquartile range of the placebo group is also shown for reference (red squares). Panel B shows the median change of the lipid ratio in each quartile with the interquartile range shown by the whiskers. The median change and interquartile range of LDL-C in the placebo group is also shown for reference (red squares).