Supplemental Materials

Variability of the Plasma Lipidome and Subclinical Coronary Atherosclerosis

Sock Hwee Tan[1,2]*, Hiromi W.L. Koh[1,3]*, Jing Yi Chua[2], Bo Burla[4], Ching Ching Ong[5], Li San Lynette Teo[5], Xiaoxun Yang[1], Peter I. Benke[4], Hyungwon Choi[1], Federico Torta[4,6], A. Mark Richards[1,2,7], Markus R. Wenk[4,6]†, and Mark Y. Chan[1,2]†

[1] Cardiovascular Research Institute, Yong Loo Lin School of Medicine, National University of Singapore

[2] National University Heart Center, Singapore

[3] Saw Swee Hock School of Public Health, National University of Singapore, Singapore,

[4] Singapore Lipidomics Incubator (SLING), Life Sciences Institute, National University of Singapore

[5] Department of Diagnostic Imaging, National University Health System, Singapore

[6] Department of Biochemistry and Precision Medicine Translational Research Programme, Yong Loo Lin School of Medicine, National University of Singapore

[7] Christchurch Heart Institute, University of Otago Christchurch, Christchurch Hospital

***** These authors contributed equally as first authors.

† These authors supervised the work equally as senior authors.

Corresponding authors:

Mark Y. Chan mark.chan@nus.edu.sg

Markus R. Wenk bchmrw@nus.edu.sg

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Expanded Materials and Methods:

Coronary Tomography Computed Angiography (CTCA)

Eighty-three participants underwent a non-contrast enhanced calcium scoring CT scan between study visits 3 and 4 (collimation, 2 x 192 x 0.6 mm; 120 kV tube voltage; automatic exposure control and a reference value of 80 mAs was used for the tube current; 3 mm slice thickness with 1.5 mm reconstruction increment). Five participants with CAC >400 Agatston Units were excluded from full CTCA. Seventy-eight participants underwent a contrast enhanced CTCA scan to evaluate the burden of total, calcified, lipid-rich and fibrotic plaques. Lipid-rich plaques are at the highest risk of erosion or rupture leading to acute coronary events, while fibrous- and calcified plaques are typically more stable and remain clinically silent[1, 2]. Participants with a heart rate >65 beats/min received an oral dose of beta-blocker (50–100 mg metoprolol) one–hour before the scan. Coronary artery vasodilation was induced with sublingual glyceryl trinitrate.

CTCA was performed on a third generation dual-source 2 x 192-slice CT (DSCT) system (Somatom Force, Siemens Medical Systems, Forchheim, Germany). The CTCA scan parameters were as follows: prospectively ECG-triggered sequential scan protocol; collimation 2 x 192 x 0.6 mm, using a flying focal spot technique and a gantry rotation time of 250 ms, CARE kV technique with a reference tube voltage of 100 kV, AEC with a reference value of 300 mAs was used for the tube current. The CTCA images were reconstructed in the cardiac phase with the least motion, using a medium sharp kernel (Bv40), with advanced modelled iterative reconstruction strength concentration 3 (ADMIRE; Siemens Medical Solutions, Forchheim, Germany), 0.75 mm slice thickness with 0.5 mm increment.

A test bolus technique was used to determine the time to peak for contrast enhancement. A 10 mL test bolus of Iohexol (Omnipaque 350, General Electric Healthcare) was injected into an antecubital fossa vein at a flow rate of 5–6 mL/s using a dual-head power injector (Medrad Stellant D, Bayer HealthCare), followed by a 50 mL saline bolus chaser. The time to peak contrast enhancement in the ascending aorta was obtained using a dynamic evaluation software, by placing a manually drawn 1–cm^2 region of interest in the ascending aorta. The time to peak contrast enhancement for the CTCA was determined by adding three seconds to the time to peak contrast enhancement at the ascending aorta. This was to ensure proper time to peak contrast enhancement at the distal coronary arteries. During the CTCA acquisition, a 50–60 mL bolus of Iohexol was injected at the same flow rate used during test bolus injection, followed by a 50–60 mL of saline bolus chaser. The contrast dose administered for the actual CTCA was adjusted to the participants' body weight (50 mL for those weighing

up to 85 kg; 55 mL for those weighing 85–100 kg; and 60 mL for those weighing more than 100 kg). Effective radiation dose was calculated by multiplying the dose-length product by 0.014 $mSv \cdot mGy^{-1} \cdot cm^{-1}$ as the constant k-value for cardiovascular imaging. The mean estimated radiation dose for each patient (calcium score and CTCA) was 2.1 mSv ± 1.1 mSv (range 0.87–8.6 mSv).

CTCA Image Analysis

The CTCA dataset were transferred to a post-processing workstation (syngo.via VB10B, Siemens, Forchheim, Germany) for further analysis. A single observer, blinded to the participants' history, analysed the coronary artery plaque characteristics, using a dedicated semi-automatic software prototype (Coronary Plaque Analysis 2.0.3 syngo.via FRONTIER, Siemens, Forchheim, Germany). This software uses automated segmentation and allows volumetric quantification and differentiation of lipid-rich, fibrous, and calcified plaques based on attenuation values. The cut-off value (Hounsfield Units (HU) for the calcified plaques was defined as 150% of the mean participant intravascular density, measured at each of the proximal coronary arteries. The cut-off values for the non-calcified plaques used by the analysis software were as follows: lipid-rich plaques (-100–30) and fibrotic plaques (30–190). All coronary artery segments with a luminal diameter of at least 2 mm were included in the analysis. The software automatically generated contours of the external vessel wall and internal lumen for each vessel. These automated contours were then carefully reviewed and manually adjusted when necessary. The software then generated the segmented vessel length, total plaque volume, calcified plaque volume, non-calcified fibrotic plaque volume and non-calcified lipid-rich plaque volume. The plaque volume was then divided by the vessel length to obtain the plaque volume index.

Lipid Extraction and Profiling

Samples were randomized into six analytical batches and batch quality control (BQC) samples were prepared by pooling equal plasma aliquots from all the samples. To assess analytical performances, one BQC sample was included for every ten study samples. Plasma (10 µL) was mixed with 90 µL 1-butanol/methanol (1:1, v/v)[3] art containing 4.5 µL of SPLASH™ Lipidomix® Mass Spec Standard I (#330707) and 4.5 µL Cer/Sph Mixture I (#LM6002) from Avanti Polar Lipids, Inc. The mixture was vortexed for 30 s, sonicated for 30 min at 20°C and then centrifuged at 14,000 x g for 10 min at 10°C. The supernatant was transferred into autosampler vials. Extracted blanks were prepared using the same extraction protocol but without plasma samples. Technical quality control (TQC) samples were generated by pooling lipid extracts from study samples. To test linear response, a dilution series was prepared by

diluting TQCs extracts with 1-butanol/methanol (1:1, v/v) at ratios 8:10, 6:10. 4:10, 2:10 and 1:10.

S1P analysis was based on Narayanaswamy et al.,[4]. Briefly, 10 µL plasma was extracted with 90 µL 1-butanol/methanol (1:1, v/v)[3] containing 20 ng/mL 13C2,D2-Sphingosine-1-phosphate d18:1 (Toronto Research Chemicals Inc. S681502) as internal standard. 90 µL extract was transferred into a 2 mL Eppendorf tube and derivatized with 10 µL 2.0M (Trimethylsilyl)diazomethane solution (Sigma-Aldrich, 527254). The tubes were capped immediately and placed into a thermomixer (Eppendorf, Thermomixer comfort) at 25°C and shaken at 700 rpm. After 30 min, 1 µL acetic acid was added to stop the reaction and neutralizing the reagent, then the derivatized samples were transferred into autosampler vials.

Liquid chromatography-mass spectrometry (LC-MS)

Targeted liquid chromatography-mass spectrometry (LC-MS)-based lipidomic analyses were performed in positive MRM mode using an Agilent 1290 Infinity II system combined with an Agilent 6495 A triple quadrupole MS in positive MRM mode. MS source parameters were: gas temperature, 200°C; gas flow 14 L/min; nebulizer: 20 psi; sheath gas heater 250°C; sheath gas flow 11 L/min; capillary voltage: 3500 V; nozzle voltage: 500 V, Delta EMV(+): 200 V, ion funnel high and low pressures RF, 150 and 60, respectively. One microliter of lipid extract was chromatographically separated using an Agilent Zorbax RRHD Eclipse Plus C18, 95Å (50 x 2.1 mm, 1.8 µm) column at 0.4 mL/min maintained at 40°C. The mobile phase consisted of (A) 10 mmol/L ammonium formate in acetonitrile/water (40:60, v/v) and (B) 10 mmol/L ammonium formate in acetonitrile/2-propanol (10:90, v/v). The gradient elution program was 20% B to 60% B from 0–5 min, 60 to 100% B from 2–7 min, where it was maintained until 9 min, and from 9.1 min, re-equilibrated at 20% B for 1.7 min prior to the next injection. The total run time was 10.8 min. TQCs were inserted at regular intervals throughout the analytical runs to monitor the instruments performance. BQC samples were used to estimate the global analytical performance (including lipid extraction) and to estimate the analytical coefficient-of-variability. All samples were kept at 7°C in the autosampler.

S1P analysis were carried out on the same instrumentation, using positive MRM mode.[5] MS source parameters were: gas temperature, 200°C; gas flow 12 L/min; nebulizer: 25 psi; sheath gas heater 400°C; sheath gas flow 12 L/min; capillary voltage: 3500 V; nozzle voltage: 500 V, Delta EMV(+): 200V, ion funnel high and low pressures RF, 200 and 110, respectively. Three microliter of lipid extract was chromatographically separated using a Waters ACQUITY BEH HILIC 130Å (100 x 2.1 mm, 1.7 µm) column at 0.4 mL/min, maintained at 60°C. The mobile phase consisted of (A) 50% acetonitrile in water containing 25 mM

ammonium formate pH 4.6 and (B) 95% acetonitrile in water containing 25 mM ammonium formate pH 4.6. The gradient elution program was 99.9% B to 40% B from 0–5 min, 40 to 10% B from 5–6.5 min and re-equilibrate at 99.9% B for 3 min. The total run time was 9.60 min. All samples were kept at 8°C in the autosampler.

Quantification of lipids and data normalization

Peak integration was performed using the MRMkit software.[6] Of all peaks integrated, we accepted a lipid measurement if the ratio of its median peak area in the BQC samples to extracted blanks was more than 10, coefficient of variation less than 30% or D-ratio less than 50%,[7] and the linear response of the TQC dilution series showed an r2 >0.8 and an y-intersect < 40% of the undiluted TQC. M+2 isotopic correction[8] was carried out for lipid species whose isotopes were co-integrated within the main species due to lack of chromatographic separation.[5] The resulting dataset consisted of 284 lipid species. The concentration data was further normalized using the nonparametric regression and batch median equalization routine built in MRMkit. For all downstream analysis, log2-transformation was applied to the corrected values and only individuals with at least three time-point measurements were used in the statistical analyses.

Supplemental Reference:

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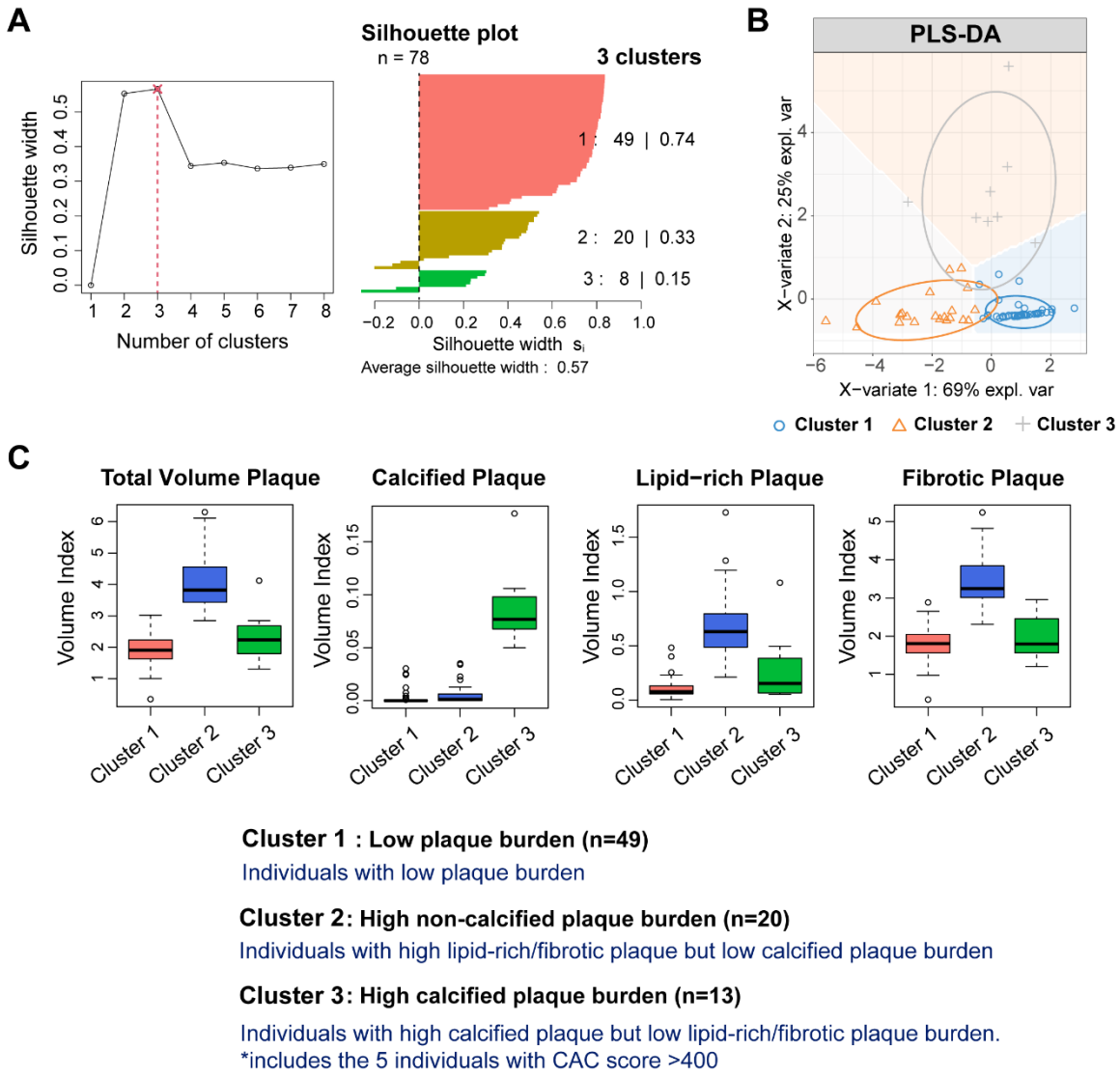
Supplemental Figure



Cluster 1 : Low plaque burden (n=49)

Individuals with low plaque burden

Cluster 2: High non-calcified plaque burden (n=20)

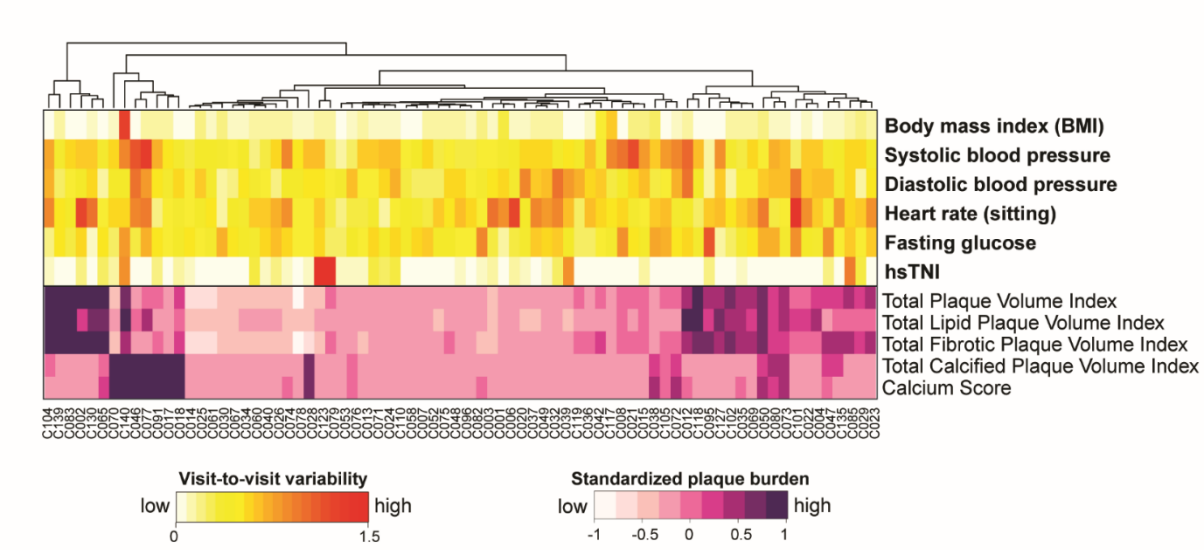
Individuals with high lipid-rich/fibrotic plaque but low calcified plaque burden

Cluster 3: High calcified plaque burden (n=13)

Individuals with high calcified plaque but low lipid-rich/fibrotic plaque burden.
*includes the 5 individuals with CAC score >400

Supplemental Figure I: Cluster analysis of 78 subjects based on calcified, lipid-rich and fibrotic volume plaque index

(A) Scree plot and silhouette plot illustrating the choice of 3 optimal clusters separating the 78 subjects. (B) Partial least squares discriminant analysis (PLS-DA) plot shows good separation among the three clusters where the first component separates cluster 1 and 2 and second component separates cluster 3 from the rest. (C) Boxplots of the individual plaque indices that characterizes the three clusters where cluster 1 (n=49) is made up of subjects with overall low plaque burden, cluster 2 (n=20) with high non-calcified plaque burden and cluster 3 (n=13) with high calcified plaque burden.



Supplemental Figure II: Visit-to-visit variability of clinical measurements on different phenotypes of plaque burden.

Heatmap of the visit-to-visit variability of body mass index (BMI), systolic and diastolic blood pressure, sitting heart rate, fasting glucose and high sensitivity troponin-I (hsTNI) measurements across the 77 individuals based on hierarchical clustering with calcium score, total plaque, lipid plaque, fibrotic plaque and calcified plaque volume index.

Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Other

Description	Catalog #	URL
SPLASH® LIPIDOMIX® Mass Spec Standard	330707	https://avantilipids.com/product/330707
Cer/Sph Mixture I (Ceramide/Sphingoid Internal Standard Mixture I)	LM6002	https://avantilipids.com/product/lm6002
D-erythro-Sphingosine-1-phosphate-13C2,D2	S681502	https://www.trc-canada.com/product-detail/?S681502
(Trimethylsilyl)diazomethane solution	527254	https://www.sigmaaldrich.com/SG/en/product/aldrich/527254