7-2024

Development and validation of a plasmalogen score as an independent modifiable marker of metabolic health: population based observational studies and a placebo-controlled cross-over study

Habtamu B. Beyene

Kevin Huynh

Tingting Wang

Sudip Paul

Michelle Cinel

See next page for additional authors

Recommended Citation

Beyene, H. B., Huynh, K., Wang, T., Paul, S., Cinel, M., Mellett, N. A., Olshansky, G., Meikle, T. G., Watts, G. F., Hung, J., Hui, J., Beilby, J., Blangero, J., Moses, E. K., Shaw, J. E., Magliano, D. J., Giles, C., & Meikle, P. J. (2024). Development and validation of a plasmalogen score as an independent modifiable marker of metabolic health: population based observational studies and a placebo-controlled cross-over study. EBioMedicine, 105, 105187. https://doi.org/10.1016/j.ebiom.2024.105187

Development and validation of a plasmalogen score as an independent modifiable marker of metabolic health: population based observational studies and a placebo-controlled cross-over study

Habtamu B. Beyene,[a,b,c,l] Kevin Huynh,[a,c,d,l] Tingting Wang,[a,c,d] Sudip Paul,[a,b] Michelle Cinel,[a] Natalie A. Mellett,[a] Gavriel Olshansky,[a] Thomas G. Meikle,[a,c] Gerald F. Watts,[e,f] Joseph Hung,[e] Jennie Hui,[g,h] John Beilby,[i] John Blangero,[j] Eric K. Moses,[i,k] Jonathan E. Shaw,[a,b] Dianna J. Magliano,[a,b] Corey Giles,[a,c,d,m,**] and Peter J. Meikle[a,b,c,d,m,*]

[a]Baker Heart and Diabetes Institute, Melbourne, VIC, Australia
[b]Faculty of Medicine, Nursing and Health Sciences, Monash University, Melbourne, VIC, Australia
[c]Baker Department of Cardiovascular Research Translation and Implementation, La Trobe University, Melbourne, VIC, Australia
[d]Baker Department of Cardiometabolic Health, Melbourne University, Melbourne, VIC, Australia
[e]Medical School, University of Western Australia, Perth, WA, Australia
[f]Cardiometabolic Service, Department of Cardiology and Internal Medicine, Royal Perth Hospital, Perth, WA, Australia
[g]PathWest Laboratory Medicine of Western Australia, Queen Elizabeth II Medical Centre, Nedlands, WA, Australia
[h]School of Population and Global Health, University of Western Australia, Crawley, WA, Australia
[i]School of Biomedical Sciences, University of Western Australia, Crawley, WA, Australia
[j]South Texas Diabetes and Obesity Institute, The University of Texas Rio Grande Valley, Brownsville, TX, USA
[k]Menzies Institute for Medical Research, University of Tasmania, Hobart, TAS, Australia

eBioMedicine
2024;105: 105187

Published Online xxx
https://doi.org/10.1016/j.ebiom.2024.105187

Summary

Background Decreased levels of circulating ethanolamine plasmalogens [PE(P)], and a concurrent increase in phosphatidylethanolamine (PE) are consistently reported in various cardiometabolic conditions. Here we devised, a plasmalogen score (Pls Score) that mirrors a metabolic signal that encompasses the levels of PE(P) and PE and captures the natural variation in circulating plasmalogens and perturbations in their metabolism associated with disease, diet, and lifestyle.

Methods We utilised, plasma lipidomes from the Australian Obesity, Diabetes and Lifestyle study (AusDiab; n = 10,339, 55% women) a nationwide cohort, to devise the Pls Score and validated this in the Busselton Health Study (BHS; n = 4,492, 56% women, serum lipidome) and in a placebo-controlled crossover trial involving Shark Liver Oil (SLO) supplementation (n = 10, 100% men). We examined the association of the Pls Score with cardiometabolic risk factors, type 2 diabetes mellitus (T2DM), cardiovascular disease and all-cause mortality (over 17 years).

Findings In a model, adjusted for age, sex and BMI, individuals in the top quintile of the Pls Score (Q5) relative to Q1 had an OR of 0.31 (95% CI 0.21–0.43), 0.39 (95% CI 0.25–0.61) and 0.42 (95% CI 0.30–0.57) for prevalent T2DM, incident T2DM and prevalent cardiovascular disease respectively, and a 34% lower mortality risk (HR = 0.66; 95% CI 0.56–0.78). Significant associations between diet and lifestyle habits and Pls Score exist and these were validated through dietary supplementation of SLO that resulted in a marked change in the Pls Score.

Interpretation The Pls Score as a measure that captures the natural variation in circulating plasmalogens, was not only inversely related to cardiometabolic risk and all-cause mortality but also associate with diet and lifestyle. Our results support the potential utility of the Pls Score as a biomarker for metabolic health and its responsiveness to dietary interventions. Further research is warranted to explore the underlying mechanisms and optimise the practical implementation of the Pls Score in clinical and population settings.

Funding National Health and Medical Research Council (NHMRC grant 233200), National Health and Medical Research Council of Australia (Project grant APP1101320), Health Promotion Foundation of Western Australia, and

*Corresponding authors. Baker Heart and Diabetes Institute, 75 Commercial Road, Melbourne, VIC, 3004, Australia.
**Corresponding author. Baker Heart and Diabetes Institute, 75 Commercial Road, Melbourne, VIC, 3004, Australia.
E-mail addresses: peter.meikle@baker.edu.au (P.J. Meikle), corey.giles@baker.edu.au (C. Giles).
[l]Co-first authors.
[m]Co-senior authors.

National Health and Medical Research Council of Australia Senior Research Fellowship (#1042095).

Keywords: Pls Score; Metabolic health; Diet; Lifestyle intervention

Research in context

Evidence before this study

Research in the field of cardiometabolic health highlights the consistent association between decreased levels of circulating ethanolamine plasmalogens (PE(P)) and an increase in phosphatidylethanolamine (PE) across various cardiometabolic conditions. However, a portable scoring system that mirrors these metabolic signals is currently lacking, hindering population-scale assessments of cardio-metabolic risk linked to perturbations in plasmalogen metabolism. Therefore, there is a critical need to develop a versatile score that reflects plasmalogen homeostasis to facilitate such assessments. Moreover, dietary interventions like shark liver oil (SLO) supplementation have been shown to influence plasmalogen levels and associated health markers, underscoring the importance of understanding the interplay between diet, plasmalogen metabolism, and cardiovascular/metabolic health for advancing knowledge on modifiable factors in this domain.

Added value of this study

In our study, we developed and validated a plasmalogen score (Pls Score), as a measure reflecting a metabolic signal that encompasses both PE(P) and PE levels, capturing the natural variation in circulating plasmalogens. Our study confirms the inverse association of the Pls Score with type 2 diabetes mellitus (T2DM), cardiovascular disease, and mortality risk independent of age, sex, and BMI. Additionally, significant associations between diet/lifestyle habits and the Pls Score were observed, and validated through SLO dietary supplementation, which notably altered the Pls Score.

Implications of all the available evidence

Our findings indicate the potential of the Pls Score as a biomarker for metabolic health and a valuable tool for assessing cardiometabolic risk and all-cause mortality. Furthermore, the associations observed between diet and the Pls Score, coupled with the significant increase in peripheral plasmalogen levels and the Pls Score following dietary SLO supplementation, highlight the potential effectiveness of targeted dietary and lifestyle interventions in modifying the Pls Score and improving metabolic health.

Introduction

Plasmalogens are a sub-class of glycerophospholipids that play a crucial role in the structure and function of biological membranes. They possess a distinctive ether linkage at the sn-1 position with a double bond between the first and second carbon (vinyl ether linkage), alongside an ester linkage at the sn-2 position. The vinyl ether linkage contributes to their unique biological activities and involvement in various physiological processes.[1] Plasmalogens exhibit remarkable structural diversity, as they comprise a wide range of species with variations in head group, fatty acid, or fatty alcohol composition that contribute to the structural and functional diversity of plasmalogens in different tissues and cellular compartments. The two main subtypes of plasmalogens are choline plasmalogens [PC(P)] and ethanolamine plasmalogens [PE(P)].[2,3] Plasmalogens are ubiquitously distributed across mammalian tissues with higher abundance in brain, heart and white blood cells[4] As structural components of biological membranes, particularly the PE(P) contribute to the formation of condensed, thick bilayers, which confer stability and reduce membrane fluidity.[5,6] This unique membrane architecture impacts various cellular processes, including receptor signalling, protein localization, and membrane trafficking. In addition to their structural contribution, plasmalogens possess important physiological properties. They act as endogenous antioxidants, providing protection against oxidative stress-induced damage to cellular membrane components[3,7–9]

Emerging evidence from metabolomic and lipidomic studies has revealed alterations in plasmalogen levels across numerous disease conditions. Notably, reduced levels of circulating plasmalogens have been reported in ageing and age-related disorders such as Alzheimer's disease, T2DM, and coronary artery diseases (CAD).[10–19] Recognizing this interplay between plasmalogen metabolism and disease pathogenesis is crucial for developing effective therapeutic strategies involving plasmalogen modulation. Indeed, interventions aimed at modulating plasmalogen levels hold promise as potential therapeutic approaches for not only the aforementioned diseases, but also chronic inflammation, and hepatic steatosis as well as metabolic diseases, including obesity and insulin resistance.[20–26] Of note, we have previously reported that, in addition to boosting plasmalogen levels, supplementation with Alkyrol (a purified Shark Liver Oil) primarily containing

mono-alkyl-diacylglycerols [TG(O)]—the plasmalogen precursors reduced the PE levels and yielded potential health benefits such as anti-inflammatory properties, and amelioration of dyslipidaemia.[24] These findings have prompted investigations into the development of a comprehensive marker that captures the perturbations in plasmalogen metabolism associated with metabolic health.

Deficiency in plasmalogens such as PE(P) reported in neurodegenerative and cardiometabolic diseases is often accompanied by increased levels of PE to maintain membrane phosphatidylethanolamine level.[27,28] This compensatory response underscores the significance of plasmalogen homeostasis in maintaining metabolic balance. To effectively evaluate the cardio-metabolic risk associated with altered plasmalogen metabolism at a population scale, it is essential to develop an adaptable score that reflects plasmalogen homeostasis. The utilization of compositional data—considering the relative abundance of PE and PE(P) species—enables the derivation of informative scores through techniques such as principal component analysis (PCA). Along with other related indices, such as total concentration of PE, PE(P), PE(P)/PE ratio, and total plasmalogen, the first principal component of compositional data provides valuable insights into various aspects of plasmalogen metabolism. These include understanding plasmalogen dynamics and identifying individuals with impaired plasmalogen metabolism within a population, who may benefit the most from intervention. These markers provide a framework to explore the potential of plasmalogens as independent and modifiable markers of metabolic health.

In the present study, we developed a plasmalogen score (Pls Score) which would be an independent and modifiable marker of metabolic health, and tested whether this score was modifiable through dietary supplementation. We leveraged three independent lipidomic datasets: the Australian Diabetes, Obesity and Lifestyle Study (AusDiab, n = 10,339), the Busselton Health Study (BHS, n = 4492) and a placebo-controlled crossover study investigating the impact of dietary supplementation with Alkyrol® (a purified shark liver oil). The Pls Score represents the first principal component of the PCA performed on the compositional data of PE and PE(P) species. In addition to the Pls Score, indices such as total PE, PE(P), PE(P)/PE ratio, PE(P) normalised to total phospholipid content, total plasmalogen, and total plasmalogen as a percentage of total plasma phospholipids were calculated. By considering these indices, we sought to capture the overall representation of plasmalogen levels in circulation. Subsequently, we investigated the association between the Pls Score and cardio-metabolic risk factors and disease outcomes including T2DM, cardiovascular disease (CVD), and all-cause mortality as well as diet and lifestyle factors. We further demonstrate direct modification of the Pls Score and reduction of dyslipidaemia and inflammatory markers, using dietary supplementation of SLO. This demonstrates the ability to effectively modulate the Pls Score for health benefit.

Methods

The Australian Diabetes, obesity and lifestyle study (AusDiab)

The AusDiab cohort is a national population-based prospective study, established in 1990/2000 to study the prevalence and risk factors of diabetes and CVD in an Australian adult population. The baseline survey (conducted in 1999/2000), involved 11,247 participants aged ≥25 years old, randomly selected from the six states and the Northern Territory, comprising 42 urban and rural areas of Australia. The sample size was determined to ensure precise estimates in identifying a national diabetes prevalence of 7.0%. This estimation was derived from the findings of prior surveys, taking into account the anticipated increase in the diabetes rate over time. The detailed description of study population, methods, and response rates of the AusDiab study has been published.[29] Measurement of clinical lipids including fasting serum total cholesterol, high density cholesterol (HDL-C), and triglycerides as well as height, weight, body mass index (BMI), and other behavioural risk factors has been described previously.[30] We utilised all the baseline fasting plasma samples (n = 10,339) after excluding samples from pregnant women (n = 21), those with missing data (n = 277), technical errors (n = 19), or whose fasting plasma samples were unavailable (n = 591). The mean (SD) age of participants was 51.3 (14.3) years. Women comprise 55% of the cohort (Table 1).

The Busselton Health Study

The Busselton Health Study (BHS) is a community-based study in the town of Busselton, Western Australia; the participants are predominantly of European origin. A total of 4492 subjects in the 1994/95 survey of the ongoing epidemiological study were included. The BHS cohort was used as a validation cohort. The mean (SD) age was 50.8 (17.4) years with women constituting 56% of the cohort. The characteristics of study participants are shown in Table 1. The details of the study and measurements for HDL-C, low density cholesterol (LDL-C), triglycerides, total cholesterol, and BMI have been previously described.[31,32]

SLO supplementation study

This was a double-blind, placebo-controlled crossover study, among overweight or obese males (BMI 28–40 kg/m^2) (n = 10), aged 25–60 years, with no signs of cardiovascular disease or diabetes. The study was conducted after a written informed consent was obtained from all study participants. Participants were randomised into placebo or treatment arms and received 4-g Alkyrol® (purified SLO; Eurohealth,

Characteristic	AusDiab study (n = 10339)	BHS (n = 4492)	SLO study (n = 10)
Demographics			
Age (years)	51.3 (14.3)	50.8 (17.4)	50 (10)
Sex			
Men	4654 (45%)	1976 (44%)	10 (100%)
Women	5685 (55%)	2516 (56%)	–
BMI (kg/m^2)	26.9 (4.9)	26.2 (4.2)	32.1 (3.2)
WC (cm)	90.8 (13.8)	86.1 (12.7)	105.7 (9.4)
Clinical lipids and cardiometabolic risk factors			
Total cholesterol (mmol/L)	5.7 (1.1)	5.6 (1.1)	5.39 (1.19)
HDL-C (mmol/L)	1.44 (0.4)	1.39 (0.39)	1.09 (0.12)
Triglycerides (mmol/L)[a]	1.28 (0.9)	1.18 (0.90)	2.14 (1.08)
SBP (mmHg)	129.2 (18.6)	124.0 (17.9)	116 (13)
DBP (mmHg)	70.0 (11.7)	74.5 (10.2)	74 (10)
FPG (mmol/L)	5.3 (1.1)	5.0 (1.4)	4.9 (0.48)
2 h-PLG (mmol/L)	6.3 (2.7)	–	–
HbA1C (%)	5.2 (0.6)	–	–
HOMA-IR[a]	3.0 (2.0)	1.78 (2.5)	–
Current smoking n (%)	1623 (15.9)	608 (13.5)	–
Medications			
BP treatment n (%)	1577 (15.3)	–	–
Lipid lowering medication n (%)	871 (8.4)	108 (2.4)	–
Prevalence of outcomes			
Over all T2DM (known and new) n (%)	686 (6.6)	162 (3.6)	–
KDM n (%)	291 (2.8)	68 (1.5)	
New DM n (%)	295 (3.8)	94 (2.1)	–
Baseline CVD prevalence n (%)	577 (5.6)	238 (5.3)	–

Values in the table expressed as mean (±SD) for continuous variables or as n (%) for dichotomous variables. [a]Values expressed in median (IQR). Data for HOMA-IR is only available on people aged over 35 years, n = 8871). Abbreviations: BMI, body mass index; WC, waist circumference; HDL-C, high density cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; 2 h-PLG, 2-h post load glucose; HbA1C, glycated hemoglobin A1C; HOMA-IR, homeostatic model assessment of insulin resistance; BP, blood pressure; CVD, cardiovascular disease; AusDiab, Australian Diabetes, Obesity and Lifestyle Study; BHS, Busselton Health Study; SLO, shark liver oil.

Table 1: **Participant characteristics in the AusDiab and Busselton Health Studies.**

Ireland) per day or placebo (Methylcellulose) for 3 weeks, followed by a 3-week washout phase, and were then crossed over to 3 weeks of the alternate placebo/Alkyrol® treatment. Alkyrol®/SLO is a commercially available supplementation primarily composed of mono-alkyl-diacylglycerol [TG(O)] species. Our laboratory has previously reported that in addition to the expected increase in alkyl-diacylglycerol [TG(O)] species, circulating levels of ether phospholipids were increased significantly after 3 weeks of SLO supplementation, including the alkyl phosphatidylethanolamine [PE(O)], and alkenyl phosphatidylethanolamine [PE(P)].[24,33] Building upon this, we tested whether SLO treatment modified the Pls Score. Further details about the study design have been described previously.[24] The baseline characteristics of participants in the SLO supplementation study are presented in Table 1.

Biochemical, diet and lifestyle data measurements

The demographic and behavioural data collection has been described in detail for the AusDiab study,[29,34] the BHS[32] and the SLO supplementation study.[24] Fasting clinical lipid measures, including total cholesterol, HDL-C, LDL-C and triglycerides FPG and 2 h post load glucose (2 h-PLG) were measured using standard protocols.[35] Methods for assessment of dietary intake, exercise, smoking and television (TV) viewing time are provided in Supplementary Material S1.

Clinical outcome ascertainment

In the AusDiab cohort, diabetes diagnosis was ascertained using the WHO criteria; fasting plasma glucose (FPG) ≥7.0 mmol/L or 2 h post-load glucose (2 h-PLG) ≥11.1 mmol/L).[36] Both newly diagnosed prevalent T2DM (n = 395 cases versus 7733 normal glucose tolerance (NGT)) and 5-year incident T2DM (n = 218/5354 controls) were included. Participants with newly diagnosed prevalent T2DM were those who were not taking pharmacological treatment for diabetes, nor previously diagnosed with diabetes, and who had FPG or 2 h-PLG measurements over the diabetes cut-off. Participants were classified as having impaired fasting

glucose (IFG), if FPG was 6.1–6.9 mmol/L and 2 h-PLG was <7.8 mmol/L and impaired glucose tolerance (IGT) if FPG <7.0 and 2 h-PLG was 7.8–11.0 mmol/L. The detailed diagnostic criteria for the presence of diabetes and pre-diabetes has been reported.[37] In the AusDiab cohort, a total of 577 cases of prevalent CVD were identified by self-report. These cases encompassed a combination of individuals with a history of heart attack and stroke, as well as individuals with ischemic heart disease (IHD). The definition of IHD included angina pectoris, myocardial infarction, coronary artery bypass grafting (CABG), and percutaneous transluminal coronary angioplasty (PTCA). The identification of incident CVD and IHD cases was carried out using the international classification of diseases (ICD) codes. The cohort data was linked to National Death Index and medical records were accessed for all those who reported a CVD event, and then adjudicated those records to determine if there was an event or not. The detailed baseline characteristics of the AusDiab participants in the disease (T2DM or CVD) and control groups can be found in Supplementary Table S1. In the BHS cohort, there were 238 prevalent CVD cases for 4254 controls ascertained through health linkage data at baseline and 284 IHD events (including myocardial infarction, angina, coronary artery bypass grafting and percutaneous transluminal coronary angioplasty) recorded over 10 years follow up. The baseline characteristics of those who had an event and those who hadn't are summarised in Supplementary Table S2. Followed by these overall analyses, the participants' demographic, clinical, and biochemical characteristics were summarised with additional sex-disaggregated analyses and both sexes were equally represented based on self-reported by the participants (Supplementary Table S3).

Targeted lipidomic analysis

A butanol/methanol extraction method described previously[38] was used to extract lipids from human plasma (baseline fasting samples). Briefly, 10 µL of plasma was mixed with 100 µL of a 1-butanol and methanol (1:1 v/v) solution containing 5 mM ammonium formate and the internal standard mix (575 lipid species; one internal standard per lipid class) (Supplementary Table S4). The resulting mix was vortexed (10 s) and sonicated (60 min, 25 °C) in a sonic water bath. Immediately after sonication, the mix was centrifuged (16,000×g, 10 min, 20 °C). The supernatant was transferred into samples tubes containing 0.2 ml glass inserts and Teflon seals. The extracts were stored at –80 °C until analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The targeted lipidomic analysis was performed using dynamic scheduled multiple reaction monitoring on an Agilent 6490 triple quadrupole (QQQ) mass spectrometer, coupled to an Agilent 1290 series HPLC system. Reverse phase chromatography was performed using a ZORBAX eclipse plus C18 column (2.1 × 100 mm 1.8 µm, Agilent). Details of the method and chromatography gradient have been extensively described previously[39,40] and also available on our website.[41] Compared to our earlier study, we modified the methodology to enable a dual column setup (alternating column regeneration). In brief, the temperature was reduced to 45 °C from 60 °C with modifications to the chromatography to enable similar level of separation. Starting at 15% solvent B and increasing to 50% B over 2.5 min, then quickly ramping to 57% B for 0.1 min. For 6.4 min, %B was increased to 70%, then increased to 93% over 0.1 min and increased to 96% over 1.9 min. The gradient was quickly ramped up to 100% B for 0.1 min and held at 100% B for a further 0.9 min. This is a total run time of 12 min. The column is then brought back down to 15% B for 0.2 min and held for another 0.7 min prior to switching to the alternate column for running the next sample. The column that is being equilibrated is run as follows: 0.9 min of 15% B, 0.1 min increase to 100% B and held for 5 min, decreasing back to 15% B over 0.1 min and held until it is switched for the next sample. We used a 1-µL injection per sample with the following mass spectrometer conditions were used: gas temperature, 150 °C; gas flow rate, 17 L/min; nebuliser, 20 psi; sheath gas temperature, 200 °C; capillary voltage, 3500 V; and sheath gas flow, 10 L/min. Given the large sample size, samples were run across several batches, as described above. In the SLO supplementation study,[39] and BHS cohort,[39,42] lipidomic profiling was performed using the standardised methodology as described previously. Overall, 596 lipid species were quantified in the BHS cohort; 575 of which were common to the AusDiab cohort and SLO study cohort.

Quality control and data processing of lipidomic data

Integration of the chromatograms for the corresponding lipid species was performed using Agilent Mass Hunter version 8.0. Relative quantification of lipid species was determined by comparing the peak areas of each lipid in each patient sample with the relevant internal standard (Supplementary Table S4). A median centring approach was carried out to correct for batch effect using plasma quality control (PQC) samples.[43] Over 90% of the lipid species were measured with a coefficient of variation <20% and the median CV of 10.7% (based on PQC, samples) in the AusDiab cohort. Only technical outliers (n = 19) were excluded from the downstream analysis in the AusDiab. In the BHS cohort, the median %CV was 8.6% with 570 (95.6%) lipid species showing a %CV less than 20%. Of the 575 lipid species common to the study cohorts, 90 (15.7%) were PE and PE(P) species.

Calculation the Pls Score

Prior to the development the Pls Score the lipidomic datasets in the AusDiab and BHS cohorts were aligned

using the median and interquartile range (IQR) of each lipid species among apparently healthy individuals. The lipidomic data was $\log_{10}$ transformed prior to the alignment process. Briefly, we randomly selected young healthy subjects (age <40 years old, BMI 20–28, without diabetes and heart diseases, and not receiving lipid lowering medication, matched to clinical lipids) (n = 1038) of which 55% were women. The median value of each lipid for the healthy subset was subtracted from each lipid value in the original BHS data and then divided by the IQR values of the healthy subset. The resulting value was then multiplied by the IQR value of the AusDiab healthy subset matched for BHS covariates followed by the addition of the median value of the AusDiab healthy subsets. The alignment process is formulated into equations with description of each step (Supplementary Material S2). This alignment method was chosen as there was no common quality control (QC) sample for both cohorts, while the cohorts are similar in many aspects (with comparable age, BMI, and origin from same country). Consequently, the risk of introducing bias by aligning the validation cohort with the development cohort would be minimal. A Pls Score for each participant was calculated in the AusDiab discovery cohort using the PE and PE(P) compositional data i.e., each PE and PE(P) species expressed as a percentage of total PE & PE(P). A PCA was performed on the compositional data after scaling the data to unit variance. The PCA model built in the AusDiab cohort was employed to calculate the Pls Score in the BHS cohort and subsequently in the SLO supplementation trial samples. We also calculated the Pls Score independently in the BHS cohort and tested how this correlates with the Pls Score calculated using the AusDiab model. In addition to the Pls Score, six (6) additional indices were generated: total PE and total PE(P)—created as the sum of individual PE and PE(P) species; a PE(P)/PE ratio; PE(P) normalised to total phospholipid, PE(P) (%PL); total plasmalogen, PE(P) and alkenylphosphatidylcholine (PC(P)); and total plasmalogen expressed as a percent of total phospholipids, plasmalogen (%PL).

Statistics

To assess the correlation between a Pls Score and cardio-metabolic risk factors, we performed Pearson's correlation analyses including tests of significance. Before conducting Pearson's correlation analysis, we verified that data assumptions, such as linearity and normality, were met using by using scatter plots and histogram distribution plots. Binary logistic regression models were used to examine the relationship between the quintiles of the Pls Score and pre-diabetes or T2DM (both prevalent and the 5-year incident cases) adjusting for age, sex, and BMI. Further, we examined the association with prevalent CVD (in a logistic model) and 10-year incident IHD and all-cause mortality (over 17 years follow up) adjusted for age, sex, BMI, smoking, and diabetes (using a Cox proportional hazard model). In the Cox regression, we computed hazard ratios (HR) and the associated confidence intervals (age as the time scale variable) using coxph() function in the *survival* package. Multivariable linear regression analyses were performed to assess the associations between each dietary item or lifestyle habits—such as smoking and TV viewing time—(as exposure) and the Pls Score (as outcome), adjusting for age, and sex, or the same covaries with the addition of energy intake, smoking, history of diabetes and CVD. P-values were computed using a repeated measures ANOVA test, and statistically significant difference assumed at $\alpha < 0.05$ for the F value (for overall significance across all groups in the SLO study). A paired t-test (2-tailed) was used to assess whether there was a statistically significant change in circulating plasmalogen levels and/or the Pls Score after SLO supplementation in the clinical study after correction for multiple comparisons using the Tukey's method. Normality assumptions were verified using normality tests, such as the Shapiro–Wilk tests, before conducting the paired t-test and ANOVA. All statistical analyses were conducted in R version 4.2.2. A statistically significant p-value was set at an α threshold of 0.05.

Ethics

This study used datasets from the AusDiab biobank (project grant APP1101320) approved by the Alfred Human Research Ethics Committee, Melbourne, Australia (project approval number, 41/18) and the BHS cohort (informed consent obtained from all participants, and the study was approved by the University of Western Australia Human Research Ethics Committee [UWA-HREC; approval number, 608/15]). The current study was also approved by UWA HREC (RA/4/1/7894) and the Western Australian Department of Health HREC (RGS03656). Both studies were conducted in accordance with the ethical principles of the Declaration of Helsinki. No participant compensation was provided. The SLO trial study received approval from the Alfred Hospital Ethics Committee (approval number: 436/15).

Role of funders

The funding sources listed in the manuscript did not have a role in the study design, analysis, interpretation, or writing of the manuscript. The decision to submit the manuscript for publication was made solely by the authors listed.

Results

Characteristics of participants

We obtained clinical and lipidomic data from the AusDiab (n = 11,247), BHS (n = 4492) and SLO study participants (n = 10) with mean (SD) age of 51.3 (14.3), 50.8 (17.4) and 50 (10) respectively (Table 1). The baseline

characteristics of the subjects are shown in Table 1. Both sexes were equally represented in the AusDiab and BHS cohorts; self-reported study participants sex disaggregated features are disclosed in Supplementary Table S3. After excluding missing data, plasma lipidomic profiles were available for 10,339 participants within the AusDiab study and 4,492 in the BHS study. Among the 10,339 AusDiab participants, 686 (6.6%), and 577 (5.6%) had prevalent diabetes and CVD, respectively (Fig. 1, Table 1). In BHS, 238 (5.6%) had prevalent CVD (Fig. 1, Table 1). A flow diagram showing the study participants, exclusions and clinical end points is presented in Fig. 1.

Development of the Pls Score

Of the 575 plasma lipid species—representing 32 lipid classes/subclasses—profiled in each of the AusDiab and BHS cohorts (Supplementary Table S4), phospholipids constitute 61% of the total number measured lipid species within which there are 37 and 51 PE and PE(P) species, respectively. Pearson's correlation analysis demonstrated strong positive intra-class correlations for both PE and PE(P) species, while sparse negative inter-class correlations exist between several PE species with PE(P) species (Supplementary Fig. S1). A PCA performed on the PE and PE(P) compositional data (Fig. 2a) identified the two major components: PC1 and PC2 explaining 27.3% and 13.2% of the variance, respectively (Fig. 2b). A clear separation existed between the PE and PE(P) species in the loadings plot; where PE(P) species showed positive loadings, while PE species had negative loadings (Fig. 2c). PC2 showed separation of species within the PE and PE(P) classes based on the fatty acid composition (Supplementary Table S5). Certain plasmalogen species have a more significant impact on the Pls Score than others. The weightings in PC1 and PC2

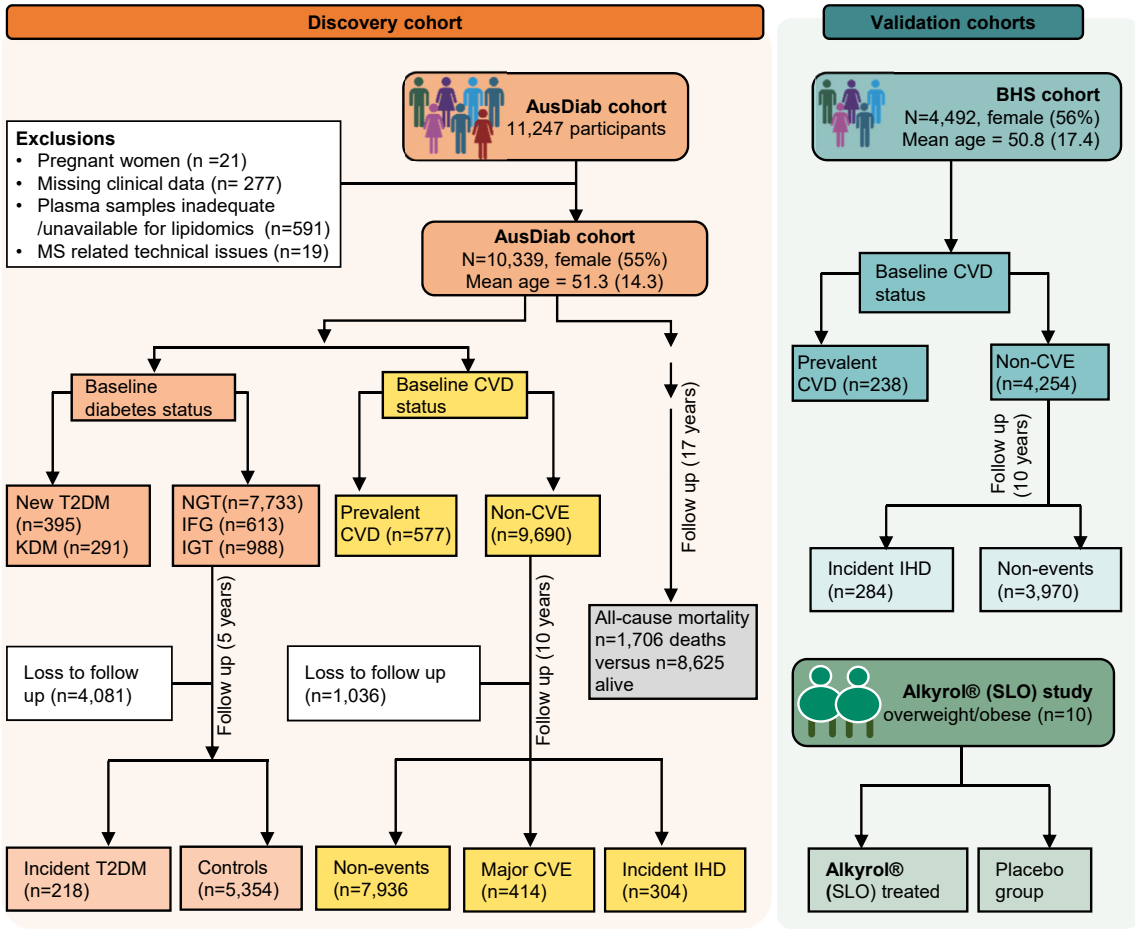

Fig. 1: Flowchart for the selection of the analysed participants from the AusDiab, BHS study cohort and SLO samples. Biobank cohort. AusDiab Australian Diabetes, Obesity and Lifestyle Study, BHS Busselton Health Study, IGT impaired glucose tolerance, IFG impaired fasting glucose, NGT normal glucose tolerance, T2DM type 2 diabetes mellitus, CVD cardiovascular disease, CVE cardiovascular event, IHD ischemic heart disease, MS, mass spectrometry; SLO, shark liver oil.

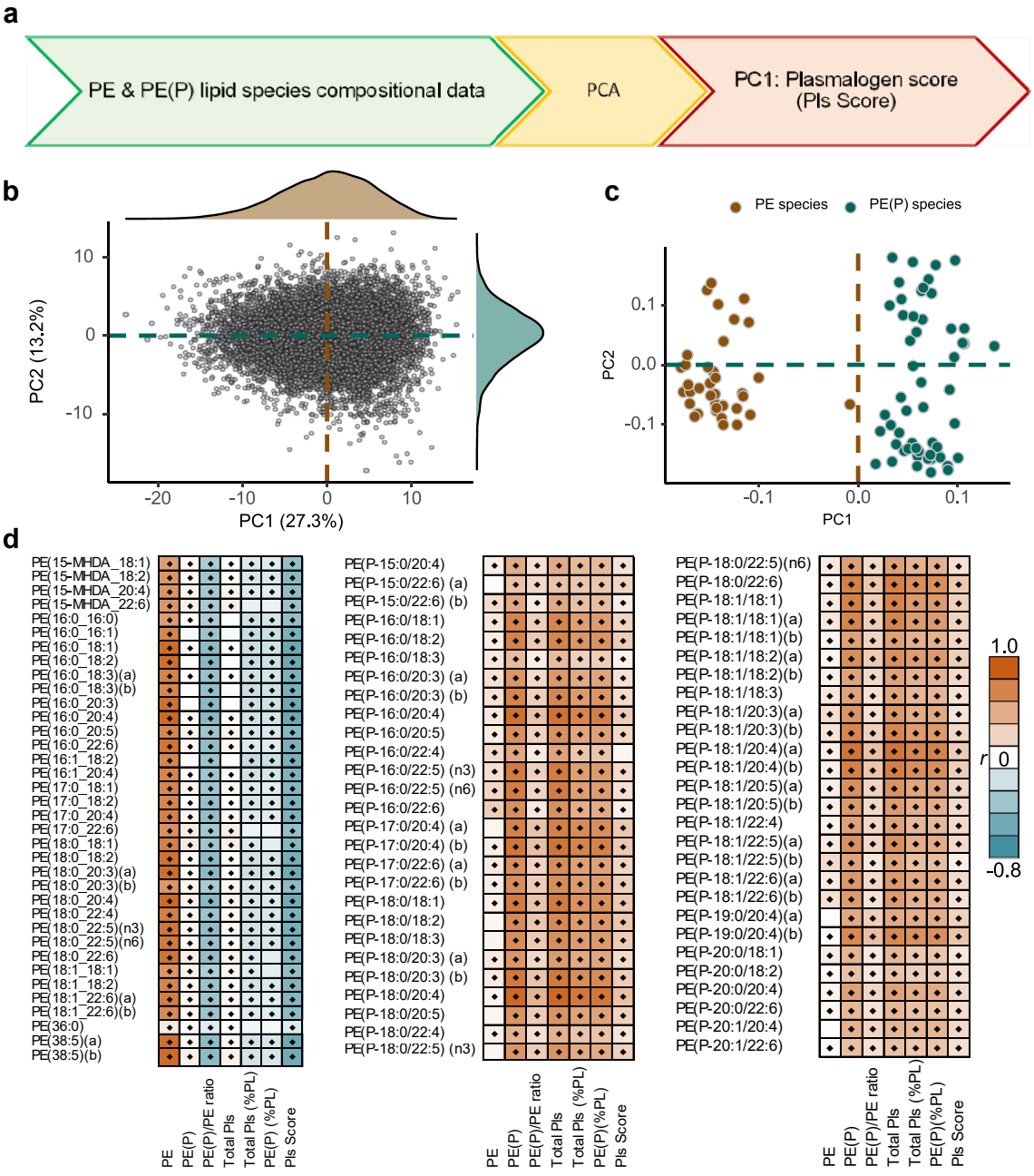

Fig. 2: An overview of the development of the Pls Score. (a) Steps involved in the Pls Score calculation: Principal components were generated using compositional data of all PE and PE(P) species (n = 91 lipids) in the AusDiab cohort (n = 10,339). (b) The scores plot of PC1 against PC2 derived from the PCA analysis. (c) A loadings plot showing the separation of PE(P) and PE lipid species. (d) The correlation heat map of the Pls Score (PC1) and related plasmalogen indices with PE and PE(P) species concentrations. Black dotted diamonds, $p < 0.05$ (Pearson's correlation). The heat maps are shaded according to the Pearson's correlation coefficients of each variable. Colour bands represent positive correlation coefficients (bronze) or negative coefficients (teal) with darker gradients depicting larger relative correlation coefficients (i.e., negative or positive). PE, phosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine; Pls, plasmalogen; PCA, principal component analysis; PC, principal components. The (a), (b), and (c) denote the elution order from chromatographic column. PE (P-17:0/22:6) (a), e.g., refers to the species that elutes prior to PE (P-17:0/22:6) (b).

for each lipid species are provided in Supplementary Table S5. We subsequently used the PC1 values as the Pls Score. Total PE, PE(P), total PE(P)/PE ratio, total PE(P) normalised to the total phospholipid [PE(P) (% PL)], total plasmalogens, and total plasmalogens normalised to the total phospholipid [total plasmalogen (% PL) were also calculated to complement the Pls Score. The correlations of the Pls Score and related plasmalogen indices with individual PE and PE(P) species are depicted in Fig. 2d. The correlation between the Pls Score and other plasmalogen indices are shown in Supplementary Fig. S2. In a regression analysis of all lipid species against the Pls Score (adjusted for age and sex), a strong negative association was observed between the Pls Score and triacylglycerol (TG), diacylglycerol (DG), ceramide, PE, lysophosphatidylethanolamine (LPE), and phosphatidylinositol (PI). As expected, ether lipids alkyl-phosphatidylcholine [PC(O)], alkenyl-phosphatidylcholine [PC(P)], lysoalkylphosphatidylcholine [LPC(O)], lysoalkenylphosphatidylcholine [LPC(P)], alkylphosphatidylethanolamine [PE(O)], and PE(P) species (Supplementary Fig. S3) were positively associated. The Pls Score model created in AusDiab was applied to the BHS cohort using PE and PE(P) compositional data (Supplementary Fig. S4). The Pls Score in BHS explained 29.4% of the variability as depicted in Supplementary Fig. S4a. The Pls Score calculated using the model developed in AusDiab was strongly correlated (R^2 = 0.997) with the score independently generated within the BHS cohort (Supplementary Fig. S4b).

The Pls Score and related indices associate with cardio-metabolic risk factors

One of the drivers for the development of the Pls Score in the present study was the need for a more holistic, independent and modifiable measure of metabolic health. We hypothesised that; the Pls Score would capture aspects of metabolic health beyond those of traditional risk factors. The Pls Score, as expected, was correlated positively with PE(P), PE(P)/PE ratio and total plasmalogens, while negatively correlated with total PE (Fig. 3). We explored the correlation between the Pls Score and related indices and different cardio-metabolic risk factors. Age (r = –0.115, p = 9.44×10^{-45}), triglycerides (r = –0.506, p = 2.2×10^{-174}), 2 h-PLG (r = –0.182, p = 9.80×10^{-85}) and SBP (r = –0.117, p = 1.94×10^{-40}) were inversely correlated with the Pls Score, while HDL-C (r = 0.110, p = 1.61×10^{-32}) was positively correlated in AusDiab (Fig. 3, bottom panel). Similar results were observed in the BHS cohort, including significant correlations between the Pls Score and cardio-metabolic risk factors (Fig. 3, upper panel).

Pls Score is significantly associated with type 2 diabetes and pre-diabetes

Reduced plasmalogen levels have been implicated in insulin resistance and T2DM pathogenesis. Lipid scores such as the phosphatidylcholine plasmalogen score (PC-PLs) have previously been implicated in T2DM.[19] However, a metabolic health score representing the most abundant plasmalogens such as PE(P) and their corresponding diacyl-phosphatidylethanolamine species is lacking. The Pls Score described here, not only serves as a tool to monitor the circulating plasmalogen levels but also as a surrogate marker for metabolic dysfunction associated with impaired glucose homeostasis and T2DM risk. To test this, we stratified the AusDiab participants into quintiles, based on their Pls Score. Logistic regression analyses were then examined for the association between the Pls Score quintiles and T2DM (prevalent and 5-year incident) or prediabetes (prevalent) stratified by isolated impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) adjusting for age, sex, and BMI). Relative to those in the lowest quintile of the Pls Score (Q1), individuals in the Q5 had an odds ratio (OR) of 0.31 (95% CI 0.21–0.43) for prevalent T2DM (Fig. 4a), and an OR of 0.39 (95% CI 0.25–0.61) for the 5-year incident T2DM (Fig. 4b). In the analysis for prediabetes, individuals were stratified into IGT and IFG, excluding individuals with concurrent IGT and IFG. Compared to those in the Q1, individuals in the Q5 had an OR of 0.48 (95% CI 0.38–0.61) for IGT (Fig. 4c) suggesting a protective association of Pls Score with isolated IGT. However, there was no significant association between the Pls Score and isolated IFG (Fig. 4d). Analyses adjusted for age, sex, BMI and clinical lipids (total serum cholesterol, HDL-C and triglycerides) can be found in Supplementary Fig. S5.

The study also examined the associations between additional Pls Score related indices, such as PE, PE(P), PE(P)/PE ratio, PE(P) (%PL), total plasmalogens, and total plasmalogens as a percentage of total plasma phospholipids, with T2DM (prevalent and incident). For PE the odds ratio associated with prevalent T2DM and incident T2DM was 1.68 (95% CI 1.49–1.88) and 1.54 (95% CI 1.33–1.78), respectively. The PE(P)/PE ratio was negatively associated with these outcomes. Detailed associations for all indices and T2DM can be found in Supplementary Table S6.

The Pls Score associates with CVD risk and all-cause mortality

To understand the role of plasmalogens in cardiometabolic health, mortality, and potentially longevity, we examined at the relationship of a Pls Score with CVD as well as all-cause mortality leveraging appropriate statistical models adjusted for potential confounders. In an age, sex, BMI, smoking, and history of diabetes adjusted analysis, a statistically significant relationship was observed between the Pls Score and prevalent CVD. Participants in the Q5, Q4, Q3 and Q2 of the Pls Score relative to Q1 had an OR of 0.42 (95% CI 0.30–0.57), 0.55 (95% CI 0.41–0.73), 0.67 (95% CI 0.51–0.86), and 0.77 (95% CI 0.60–0.98), respectively for prevalent CVD

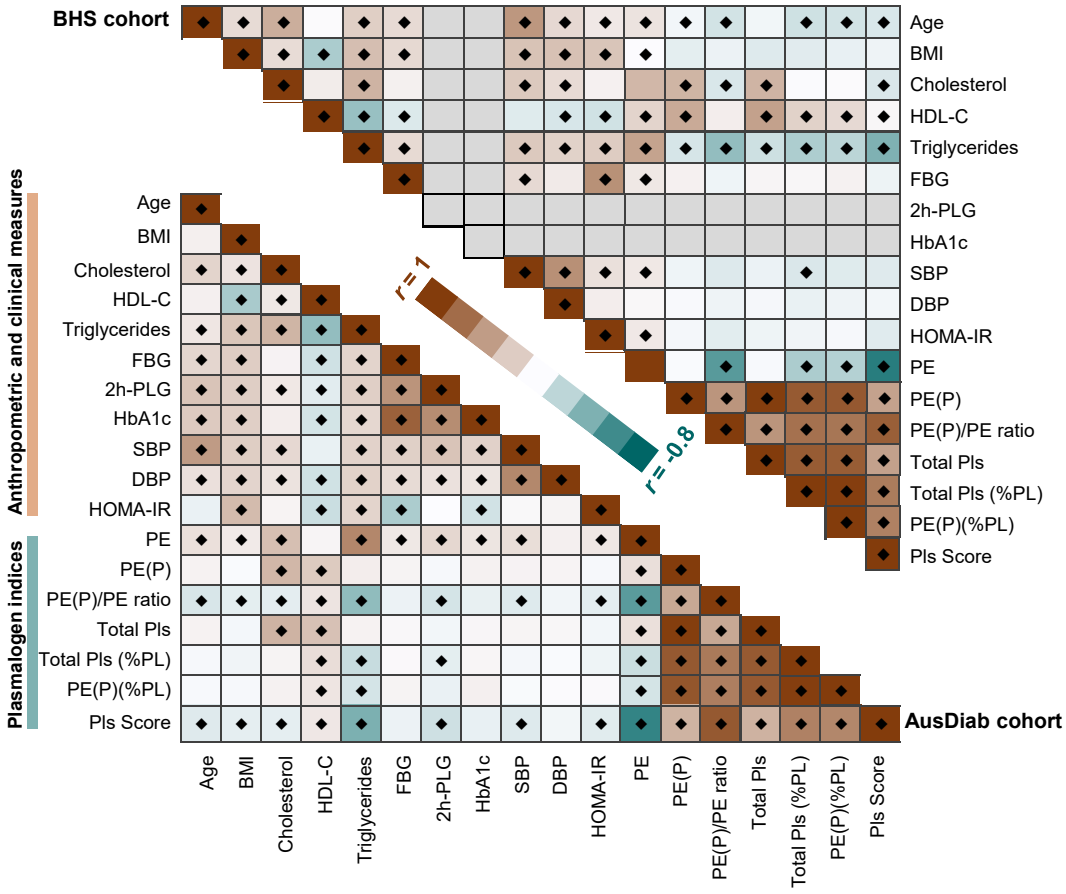

Fig. 3: Associations of the Pls Score and related indices with cardio-metabolic risk factors in the AusDiab (n = 10,339) and BHS (n = 4492) cohorts. Pearson's correlation coefficients between the Pls Score, related indices, and cardio-metabolic risk factors were computed. Statistically significant correlations ($p < 0.001$) for Pearson's correlation coefficients are indicated by black diamonds within each box. The grey squares indicate data unavailability for 2 h-PLG and HbA1c in the BHS cohort. AusDiab, the Australian Diabetes Obesity and Lifestyle Study; BHS, Busselton Health Study; BMI, body mass index, HDL-C, high density cholesterol, HOMA-IR, homeostatic model assessment of insulin resistance, FPG, fasting plasma glucose, 2 h-PLG, 2-h post load glucose, SBP, systolic blood pressure, DBP, diastolic blood pressure, HbA1C, hemoglobin A1C; Pls Score plasmalogen score; PL, phospholipid; PE, phosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine; Pls, plasmalogen.

(Fig. 5a). The association of a Pls Score with incident IHD (Cox regression with age as a time scale variable) adjusted for sex, BMI, smoking, and history of diabetes in the AusDiab was significant only in Q5 relative to Q1 (Fig. 5b). When examining the risk of all-cause mortality across the quintiles of the Pls Score, individuals in the Q5 to Q2 had significantly lower risk relative to those in the Q1, with a 34% lower risk in Q5 versus Q1 (HR = 0.66; 95% CI 0.56–0.78) (Fig. 5c). The survival plot for all-cause mortality stratified by the lowest and highest quintiles of the Pls Score in the AusDiab cohort is depicted in Fig. 5d. Analyses adjusted for age, sex, BMI, smoking, SBP, baseline diabetes status and clinical lipids (total serum cholesterol, HDL-C, and triglycerides) can be found in Supplementary Fig. S6.

PE showed a significant positive association with prevalent CVD (OR = 1.37; 95% CI 1.16–1.62) and all-cause mortality (HR = 1.13; 95% CI 1.07–1.19) (Supplementary Table S7). Conversely, PE(P) (OR = 0.85; 95% CI 0.78–0.93), PE(P)/PE ratio (OR = 0.75; 95% CI 0.68–0.83), total plasmalogen (OR = 0.83; 95% CI 0.75–0.90), and total plasmalogen (%PL) (OR = 0.90; 95% CI 0.82–0.99) were negatively associated with prevalent CVD. Similarly, PE(P) (HR = 0.92; 95% CI 0.87–0.96), PE(P)/PE ratio (HR = 0.85; 95% CI 0.81–0.90), total plasmalogen (HR = 0.92; 95% CI 0.87–0.96), and total plasmalogen (%PL) (HR = 0.92; 95% CI 0.87–0.97) showed significant negative associations with all-cause mortality in the AusDiab cohort (Supplementary Table S7).

In the BHS cohort (validation), the Pls Score, PE(P)/PE (ratio) and total plasmalogen (%PL) showed significant negative associations with prevalent CVD and incident IHD. While higher PE was associated with an increased likelihood of having prevalent CVD (OR = 1.47;

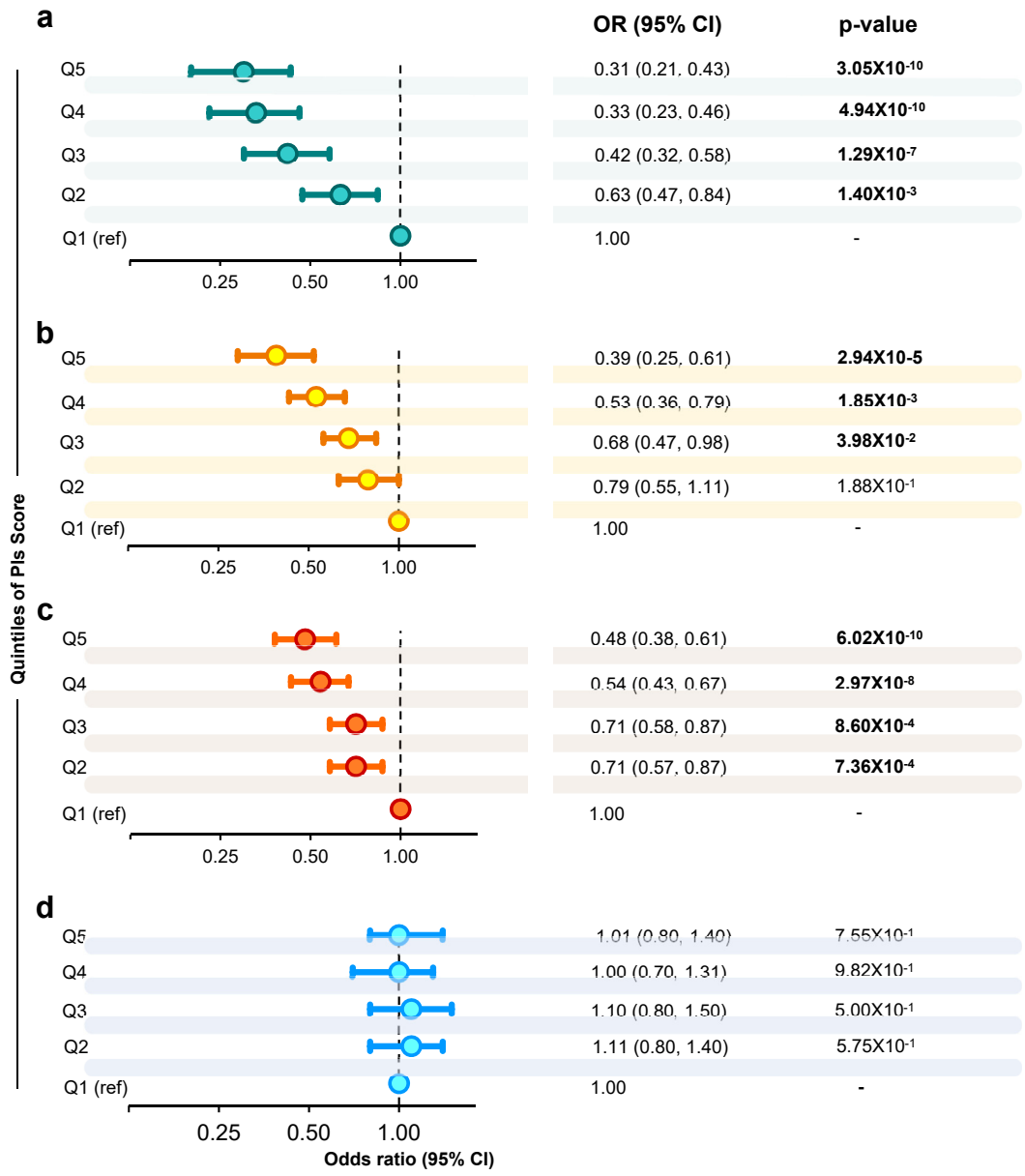

Fig. 4: Associations of Pls Score with T2DM and pre-diabetes in the AusDiab. A logistic regression analysis was performed between T2DM or prediabetes against quintiles of the Pls Score. The forest plots show the odds ratio (x-axis) associated with moving from Q1 of the Pls Score (reference quintile) to Q2–Q5 (y-axis) for the newly diagnosed prevalent T2DM (a), 5-year incident T2DM (b), isolated IGT (n = 988 versus 7733 NGT) (c), and isolated IFG (n = 613/7733 NGT) (d) compared to controls. The odds ratios were computed from multiple logistic regression analyses between the outcomes and the quintiles of the Pls Score (Q1 as a reference) adjusted for age, sex, and BMI. Error bars represent 95% CIs. The odds ratios and the associated CIs were log 2 transformed to enhance visualization. The results for clinical lipids, adjusted models are provided in Supplementary Figure S5. T2DM, type 2 diabetes mellitus; IGT, impaired glucose tolerance; IFG, impaired fasting glucose; NGT, normal glucose tolerance; Pls, plasmalogen.

95% CI 1.25–1.72) and incident IHD (HR = 1.24; 95% CI 1.09–1.41) (Supplementary Table S8). The PE(P) and total plasmalogen did not show any significant association with prevalent CVD and incident IHD in the BHS cohort (Supplementary Table S8).

Lifestyle and dietary habits associated with Pls Score

An investigation into the link between lifestyle and diet and Pls Score offers valuable insights into potential modifiable risk factors for cardiovascular and metabolic

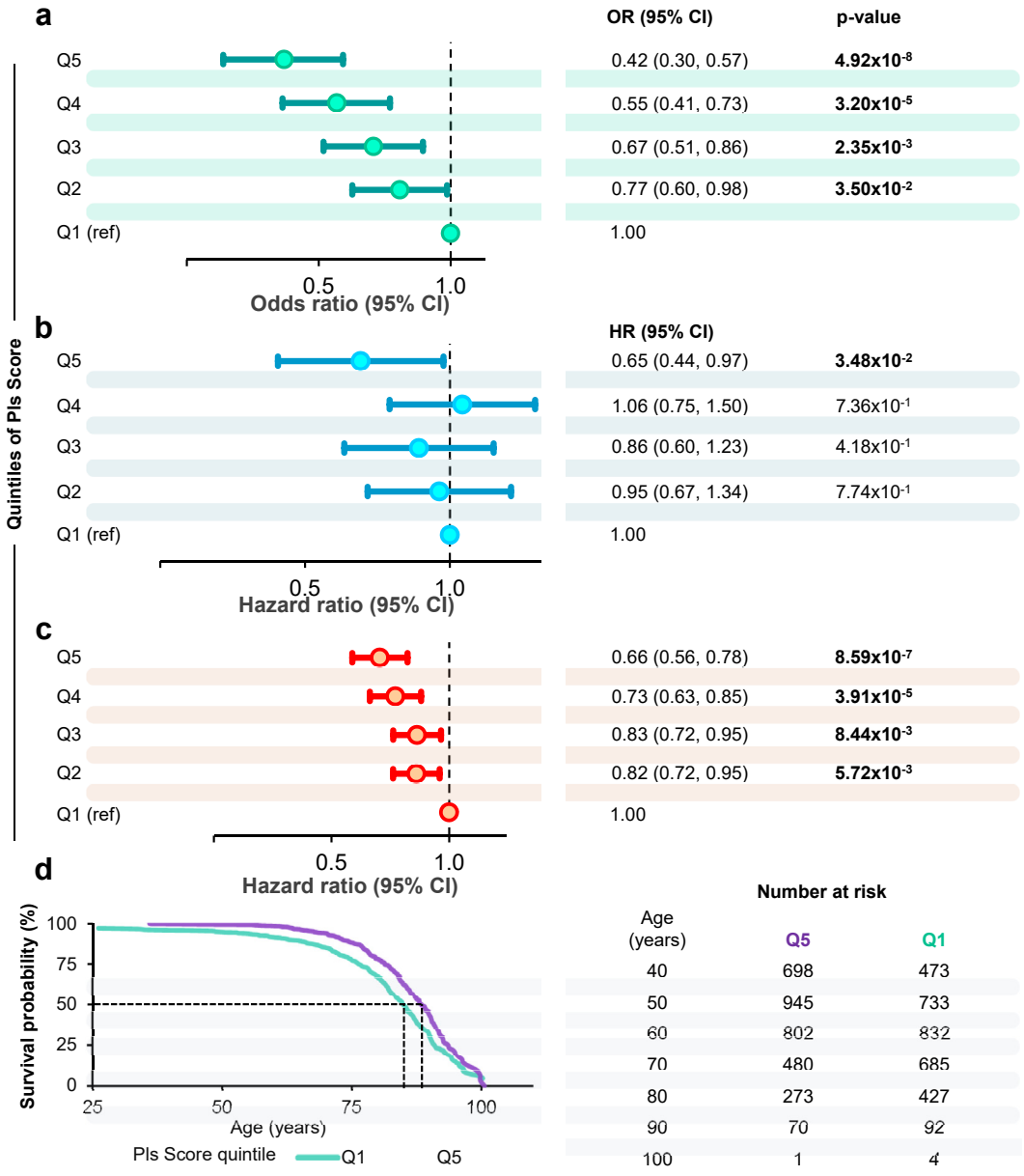

Fig. 5: Associations between Pls Score and CVD and all-cause mortality adjusted for age, sex, BMI, smoking and history of diabetes in the AusDiab cohort. The association between the quintiles of the Pls Score, with Q1 as a reference (y-axis) and (a) prevalent CVD, n = 577/9690 (logistic regression); (b) 10-year incident IHD, n = 304/8046 (proportional hazard Cox-regression); and (c) all-cause mortality (over 17 years), n = 1706/8625. (d) An unadjusted survival plot for all-cause mortality over 17 years stratified by the top (Q5) and bottom quintiles (Q1) of the Pls Score. Each circle represents the odds ratio/Hazard ratio (x-axis) and the whiskers represent 95% CI. The results further adjusted for clinical lipids are provided in Supplementary Figure S6. Error bars represent 95% CIs. Odds ratios and the associated CIs were log 2 transformed to enhance visualization.

diseases, aiding in the development of targeted interventions and prevention strategies. Here, we assessed, the relationship between daily diet intakes (including red meat, fiber, dairy, total fruit, saturated fat, monounsaturated fatty acids (MUFA), omega-3, omega-6, odd chain fatty acid, poultry, eggs, and fish among others), lifestyle habits (TV viewing time, smoking, and exercise) and the Pls Score using multivariable linear regression analysis adjusted for relevant confounders. To examine diet- Pls Score associations, three independent models were built: (1) a model adjusted for age and sex; (2) covariates in model 1 plus energy intake;

and (3) covariates in model 2 plus smoking, exercise, diabetes status, and history of CVD. In the age and sex adjusted model, red meat intake (β = 0.32; 95% CI 0.22–0.42), poultry (β = 0.32; 95% CI 0.23–0.42), nuts and seeds (β = 0.18; 95% CI 0.08–0.27), fish (β = 0.20; 95% CI 0.10–0.29) and eggs (β = 0.29; 95% CI 0.19–0.39) were positively associated with the Pls Score. Dietary fatty acid intake—stratified into saturated fatty acid (SFA), MUFA, polyunsaturated fatty acid (PUFA), odd chain fatty acids (OCFA) and total fatty acids—were also positively associated with the Pls Score (Fig. 6a) while alcohol intake was strongly negatively associated with the Pls Score (β = –0.52; 95% CI –0.62 to –0.41) (more details can be found in Supplementary Table S9). In the subsequent models adjusted for age, sex, and energy intake (Fig. 6b) or age, sex, energy intake and lifestyle factors (Fig. 6c), SFA, MUFA, red meat, dairy intakes, eggs, poultry, total fat and alcohol intake remained significantly associated (more details presented in Supplementary Table S9). Current smoker relative to non-smokers (β = –1.51 L 95% CI –1.78 to –1.25) and TV viewing time (β = –0.25; 95% CI –1.32 to –1.7) as well as BMI were inversely associated with the Pls Score. Pls Score was also associated with sex with significantly higher scores in men relative to women (β = 1.78 95% CI 1.59–1.97). Exercise time was only marginally associated (in a positive direction) (Fig. 6d).

Diet supplementation with SLO effectively modifies the Pls Score

To gain insight into the effect of dietary supplementation with SLO on plasmalogen metabolism, and hence on the Pls Score, we conducted a small double-blind cross over human trial. Previously, our laboratory reported several lipid classes/subclasses significantly changed after SLO supplementation relative to the placebo group.[24] Here we tested whether the Pls Score was also impacted. A PCA on the compositional data in the SLO study was performed and a scores plot from this analysis can be found in Supplementary Fig. S7a. The Pls Score for the SLO supplementation samples derived using the AusDiab model strongly correlated with the score independently generated using the SLO data (R^2 = 0.99%) (Supplementary Fig. S7b).

We observed significant changes in 42 ethanolamine phospholipid species including 26 PE(P) and 16 PE species ($p < 0.05$; Supplementary Table S10) after SLO supplementation. Most of the PE(P) species were increased while PE species were decreased following SLO treatment (Supplementary Table S10). We observed a significant decrease in PE (Fig. 7a) and increases in the PE(P) (Fig. 7b), PE(P)/PE ratio (Fig. 7c), total plasmalogen (Fig. 7d), total plasmalogen (%PL) (Fig. 7e), PE(P) (%PL) (Fig. 7f) and a marked rise in the Pls Score (Fig. 7g) in SLO treated individuals, relative to the placebo group. The details of all possible pairwise comparisons can be found in the Supplementary Fig. S8. The shift in the Pls Score in SLO treated individuals was calculated relative to the AusDiab population (projecting the SLO study samples on to the AusDiab cohort). The median Pls Score before SLO was –0.5, which was dramatically increased to 6.2 after SLO supplementation. This corresponds to a shift in the median value (0.7) from the 40th centile to the new median value of 5.8 (90th centile) in the AusDiab population (Fig. 7h).

Comparing the changes resulting from SLO supplementation and placebo showed the SLO supplementation resulted in a decrease in total PE (Supplementary Fig. S9a), but an increase in PE(P), PE(P)/PE ratio, total plasmalogen, total plasmalogen (%PL), total PE(P) (%PL) and the Pls Score relative to the placebo (Supplementary Fig. S9b–g).

Discussion

The present study introduces and evaluates the Pls Score in relation to cardio-metabolic risk factors and outcomes. We also explored the influence of diet and lifestyle factors, offering a novel perspective on the intricate relationship between plasmalogens, cardiovascular and metabolic health, and modifiable lifestyle factors. The Pls Score was designed to capture the balance between PE(P) and PE levels, which have been observed to exhibit contrasting associations in various disease conditions. We demonstrate the following key findings: (1) the Pls Score captures a metabolic signal that closely mirrors the circulating levels of PE(P) and PE; (2) a strong inverse association was observed between the Pls Score and cardiometabolic diseases, including T2DM, 5-year incident T2DM, prevalent CVD, and all-cause mortality; (3) the Pls Score is associated with diet and lifestyle factors; (4) SLO supplementation markedly increased the Pls Score, further supporting the notion that dietary interventions can modify plasmalogen metabolism and potentially improve metabolic health outcomes.

The decision to utilise phosphatidylethanolamine (PE) and ethanolamine plasmalogen [PE(P)] species to devise the Pls Score in the current study was based on their observed opposing associations with various disease conditions,[17,18,28,44] although there appears to a sparse relationship in plasma level of PE and PE(P) species. Previous studies have demonstrated distinct roles for PE and PE(P) in different pathophysiological conditions, suggesting that these lipid classes may have independent effects on health outcomes. Of note, a recent report by Hornburg et al., revealed that ether-linked PE–the [PE(P)], as opposed to PE were associated with reduced steady state plasma glucose and insulin sensitivity.[17] In the context of T2DM, increased levels of PE have been consistently reported.[18,44–46] Conversely, decreased levels of PE(P) plasmalogen have been linked to increased risk,

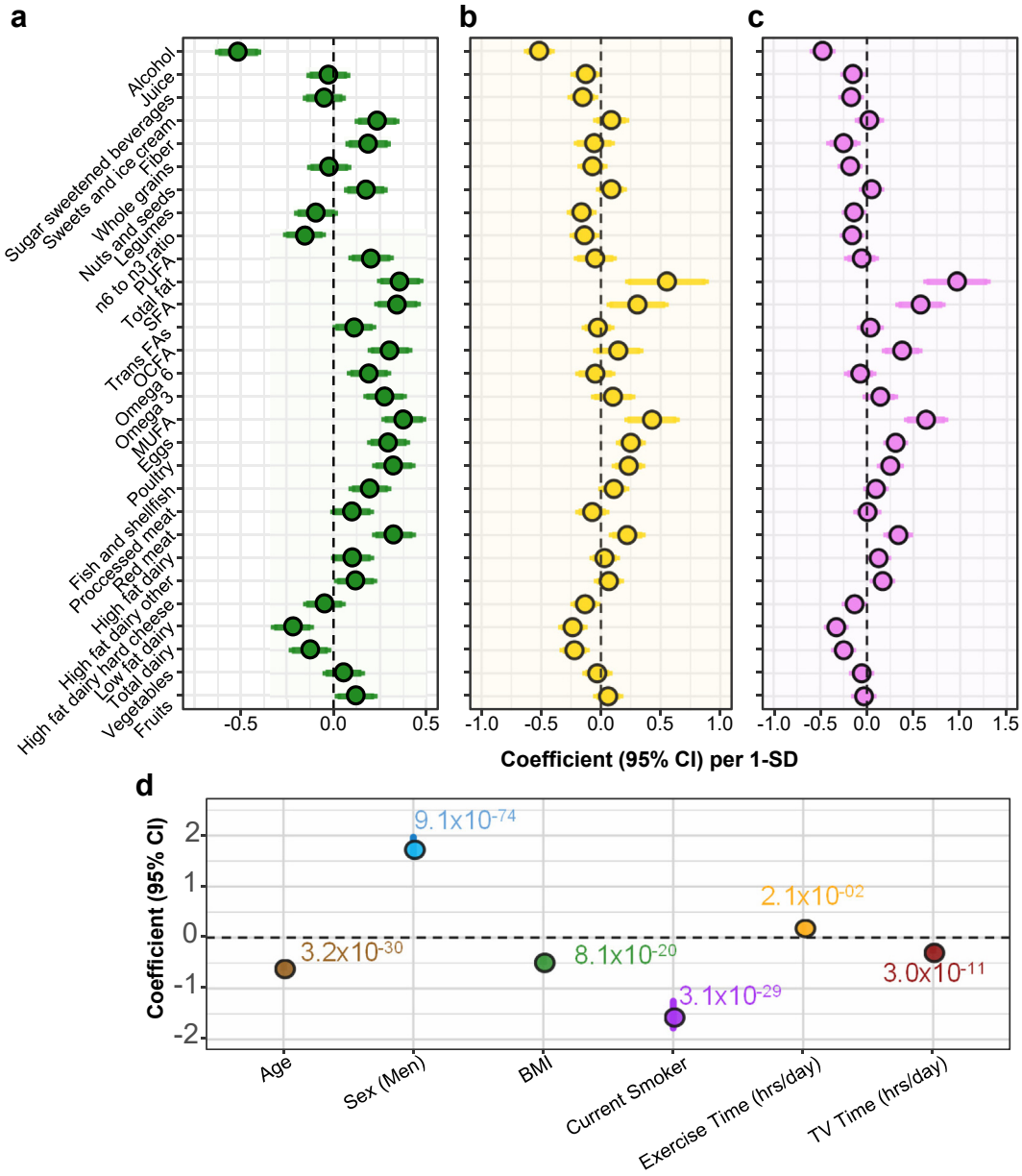

Fig. 6: The relationship between diet and lifestyle factors and the Pls Score in the AusDiab cohort (n = 10,339). A multiple linear regression analysis was performed between each dietary component (g/day intake) scaled to unit SD or lifestyle component (as exposure) and the Pls Score (outcome). Association between dietary components and a Pls Score in a model adjusted for age and sex (a); age, sex, and total energy intake (b); and age, sex, energy, smoking, exercise, diabetes, and history of CVD (c). (d) Associations between lifestyle (smoking, exercise, and TV time) and the Pls Score, adjusted for age and sex. Each circle represents the beta coefficient, and the whiskers represent 95% CI.

particularly in impaired glucose tolerance.[18,39,47] Furthermore, in conditions such as CAD and Alzheimer's disease (AD) reduced plasmalogen levels, alongside an increase in–or negligible alterations in- PE, have been reported.[16,21,28,48–50] These observations suggest that PE and PE(P) may have distinct roles in the context of cardiometabolic and neurodegenerative diseases. While higher levels of PE(P) are associated with health a persistently low levels of these particular PEs may have detrimental effects.

The Pls Score developed in this study captures the composite variation of PE and PE(P) plasmalogen species, normalised to their total abundance. It is calculated as the first principal component, which accounts for the largest proportion of variance in the combined PE and PE(P) data. As such, higher scores correspond to higher

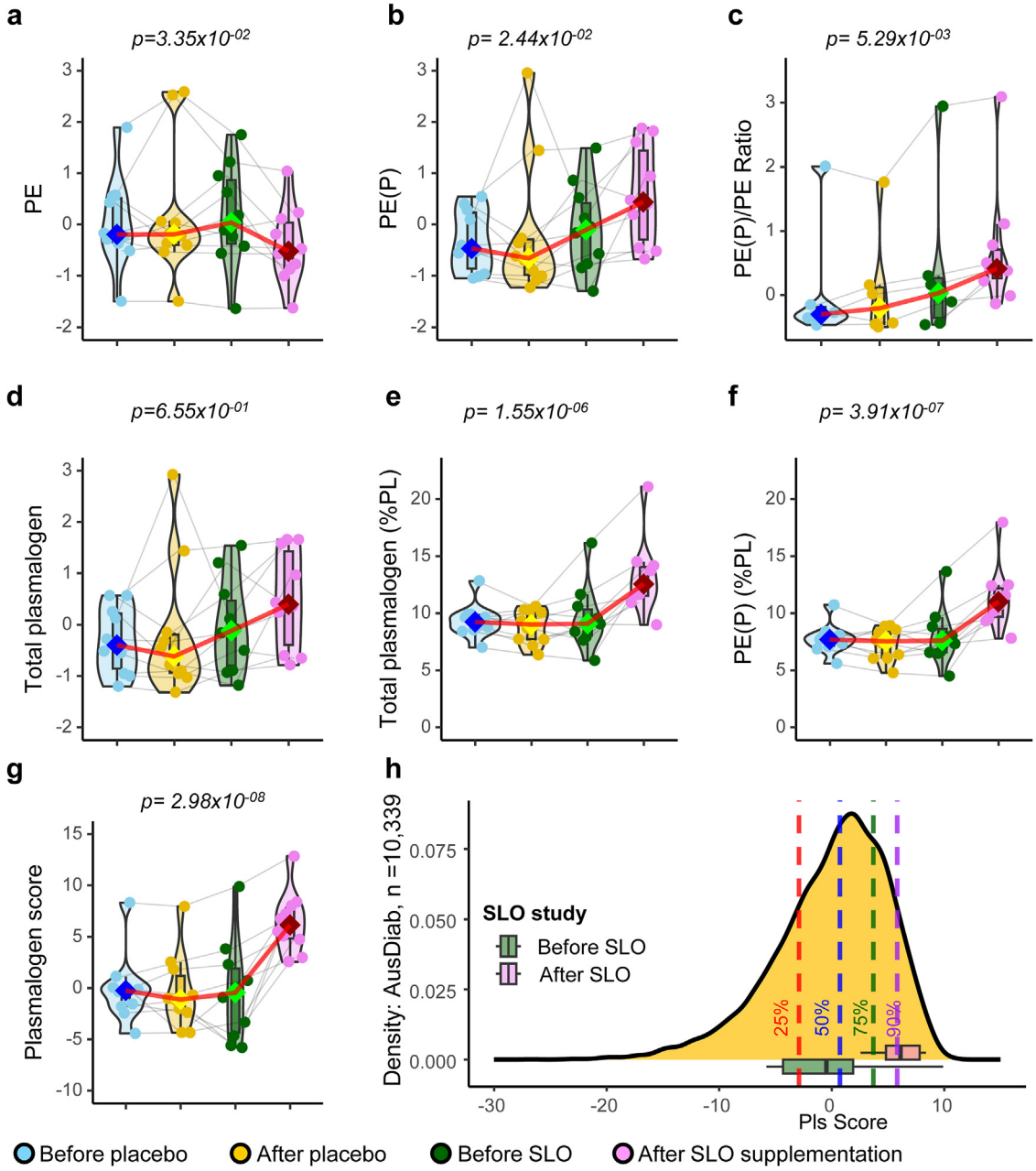

Fig. 7: The effect of SLO supplementation on plasmalogens and the Pls Score in double-blind cross over study (n = 10 obese men). SLO supplementation resulted in a decrease in PE (a), and an increase in PE(P), PE(P)/PE ratio, total plasmalogen, total plasmalogen (%PL), PE(P) (% PL) and the Pls Score (b–g). Y-axis values in panels a, b, c and d are scaled to mean of 0 and a standard deviation of 1. Gaussian distribution assumptions were checked before applying the paired t-test and ANOVA. P-values were computed using a repeated measures ANOVA test, and statistically significant difference assumed at $\alpha < 0.05$ for the F value. The data depicted in the violin box and whisker plots span from the minimum to the maximum data points. The lower and upper boundaries of the box correspond to the 25th and 75th percentiles, respectively, and the central diamonds within the boxes represent the median values. An overlay of the SLO study samples over the AusDiab; the shift in the Pls Score in SLO treated individuals relative to placebo group and the corresponding shift in the population (h).

levels of PE(P) relative to PE species. The Pls Score showed the strongest correlation with the PE(P)/PE ratio, followed by total PE (negative correlation). Additionally, Pls Score demonstrates a significant correlation with total plasmalogen and PE(P) plasmalogens normalised to the total plasma phospholipid, further

supporting its ability to capture a comprehensive plasmalogen profile. These findings highlight the utility of the Pls Score as a composite measure that integrates the relative proportions of PE and PE(P) species, providing valuable insights into plasmalogen dynamics and their relationships with other plasmalogen related indices.

In both the AusDiab and BHS cohorts, our analysis revealed statistically significant correlations between the Pls Score, along with other related indices, and various cardio-metabolic risk factors. Specifically, we report a positive correlation of the Pls Score and total plasmalogen with HDL-C and a negative correlation between the Pls Score and triglycerides. This observation aligns with earlier studies[51–53] that have reported similar findings, highlighting the positive relationship between circulating plasmalogen levels and HDL-C, as well as the beneficial effect of plasmalogens on lipoprotein metabolism. The Pls Score and the PE(P)/PE ratio are both negatively associated with age. We have previously reported that age was negatively associated with PE(P) plasmalogens, particularly those containing a 22:4 fatty acid.[54] A decline in peroxisomal metabolism and biogenesis with advancing age[55] could be one factor contributing to the decrease in plasmalogens with ageing. These insights contribute to our understanding of the complex interplay between plasmalogens, ageing, and metabolic health.

One of the key findings of our study is the significant inverse association between the Pls Score and T2DM (both prevalence and incident). Individuals with higher Pls Score scores had a reduced likelihood of having or developing T2DM. This association underscores the potential of the Pls Score as a valuable biomarker for assessing metabolic health. The role of plasmalogens in the pathogenesis of T2DM is supported by previous research linking these lipid species to the protective associations with insulin resistance, T2DM and atherosclerosis.[19,56,57] Our study also revealed a significant association between the Pls Score and IGT, but not isolated IFG. This finding highlights the different pathophysiological mechanisms of IGT and IFG suggesting a role for plasmalogens in the former. Further investigations are warranted to elucidate the underlying mechanisms and the specific contributions of plasmalogens to the development of different prediabetic states.

We report a significant inverse association between the Pls Score and prevalent CVD, as well as incident IHD, highlighting the potential cardioprotective effects of plasmalogens. The Pls Score showed a dose-dependent relationship with CVD risk, with individuals in higher quintiles having progressively lower odds of prevalent CVD. This trend was consistent in both the AusDiab and BHS cohorts, showing strong validation of these associations. A decline in ethanolamine plasmalogens[16,48,58] and increase in PE were demonstrated in CAD previously.[58,59] The distinctive characteristics of plasmalogens enable them to function as antioxidants, effectively scavenging reactive oxygen species. Moreover, plasmalogens are known to be consumed during chronic inflammatory processes. The antioxidative properties of plasmalogens and their involvement in mitigating oxidative stress and inflammation may contribute to their protective effect against T2DM and CVD. Indeed, these findings provide important insights into the role of the Pls Score in cardiometabolic risk assessment and suggest the potential of plasmalogens as targets for therapeutic interventions aimed at preventing or managing cardiometabolic diseases. A similar metric, the Omega-3 Index (tissue EPA + DHA content expressed as a percent of total fatty acids) has been associated with reduced CVD risk.[60,61] The Omega-3 Index satisfies various criteria expected of a biomarker, which include its association with disease independent of conventional risk factors, potential for modification, and, notably, the proven ability to lower the risk of CVD events by elevating tissue levels.[61] Indeed, the Pls Score and Omega-3 Index might complement each other, providing a holistic view of cardiovascular health.

Plasmalogens have been the focus of numerous studies exploring their potential role in ageing and lifespan.[17,56,62,63] Our finding of a significant association between the Pls Score and all-cause mortality further supports the notion that plasmalogens may have a beneficial impact on longevity. Individuals in the highest quintile of the Pls Score demonstrated a reduced mortality risk compared to those in the lowest quintile. Consistent with our findings, a recent study has demonstrated that lipids characterised by numerous double bonds, notably plasmalogens containing PUFAs, were negatively associated with mortality risk while positively associated with longevity.[63] Previous lipidomic studies suggest that the unique structural properties of plasmalogens, including their vinyl ether linkage, confer increased resistance to oxidative stress and this may contribute to enhanced cellular integrity and longevity.[56,62,64] However, no former study has investigated whether lower plasma ethanolamine plasmalogens levels in particular are associated with early death, although, certain alkenyl forms of phosphatidylethanolamine had been implicated in human subjects of exceptional longevity and healthy phenotype.[56] The significant association between the Pls Score and all-cause mortality provides evidence for the potential influence of plasmalogens on overall health and suggests their utility as biomarkers of longevity. However, further research is required to fully understand the specific biological mechanisms that drive these associations and to explore the therapeutic opportunities associated with modulating plasmalogen metabolism for the promotion of healthy ageing and the extension of lifespan.

Understanding the link between diet and lifestyle and the Pls Score is crucial for developing strategies to improve cardiometabolic health. Our study indicates

that higher intakes of red meat, poultry, eggs, and fatty acids (SFA, MUFA, n-3, and OCFA) are associated with a higher Pls Score. Livestock, such as cattle and poultry, as well as fish and shellfish, has been identified as a significant reservoir of plasmalogens.[65–67] Of note, high levels of complex structural lipids, such as ethanolamine plasmalogens, has been reported in chicken eggs,[65,68] fish, and molluscs[57,65] suggesting that their consumption can play a role in the synthesis and regulation of plasmalogens levels in humans. The cardioprotective mechanisms of n-3 fatty acids particularly EPA include reducing triglyceride-rich lipoproteins, incorporation into vascular membranes bolstering resistance to oxidation, improving membrane function, and providing precursors for the synthesis of specialised pro-resolving mediators that can combat inflammation.[69] However, the positive association between n-3, as well as SAF, MUFA, and OCFA with the Pls Score may relate more to these measures being colinear with total phospholipid intake, rather than a specific role of these fatty acids in plasmalogen synthesis. SFA and MUFA are typically found in animal sources, such as red meat, poultry, and eggs, while PUFA are commonly found in plant oils, nuts, seeds, and fish. These findings highlight the impact of dietary choices on plasmalogen metabolism and health. Identifying diet components that promote plasmalogen synthesis can help tailor dietary plans to enhance plasmalogen levels and reduce associated health risks.

Smoking and sedentary behaviours, as represented by TV viewing time, were negatively associated with the Pls Score, while exercise time showed a marginal positive association. Smoking has been linked to plasmalogen-deficiency characterised by reduced or insufficient activity of the peroxisomal enzyme alkyl-DHAP,[70] in addition to inducing atherogenic lipid profiles.[71] The negative association between TV viewing time and the Pls Score suggests that sedentary behaviour may contribute to a lowered plasmalogen level leading to a higher susceptibility to inflammatory diseases. Indeed, numerous studies have demonstrated that sedentary behaviour, such as prolonged TV viewing time, is associated with higher risk of inflammatory related conditions such as T2DM, CVD and mortality.[72–74] These associations emphasise the importance of healthy lifestyle choices in maintaining optimal plasmalogen levels and overall metabolic health. Further research is needed to explore the underlying mechanisms and establish causality between the lifestyle factors, plasmalogen metabolism, and health outcomes.

The plasmalogen biosynthetic pathway represents a promising target for dietary intervention due to its crucial role in maintaining cellular health and overall well-being. Plasmalogen precursors such as alkylglycerols, have often been utilised to circumvent the rate-limiting peroxisomal steps of plasmalogen synthesis and this strategy leads to elevated levels of endogenous plasmalogens by undergoing multiple enzyme-catalysed reactions within the endoplasmic reticulum.[24,33,75,76] Previously, we have shown that SLO supplementation led to a notable reduction in plasma concentrations of total free cholesterol, triglycerides, and C-reactive protein.[24] The PE and PE(P) showed distinct responses to SLO supplementation (i.e., SLO supplementation resulted in a decrease in PE, while PE(P) increased). Moreover, an abrupt shift in the Pls Score (positive direction) and related indices, such as PE(P) normalised to total phospholipids, were observed after SLO supplementation. This suggests that SLO supplementation influences the composition of phospholipids, favouring the accumulation of PE(P) while reducing PE abundance and hence contributing to an elevated Pls Score. SLO supplementation not only increases plasmalogen levels, but also involve in mitochondrial dynamics. Plasmalogen precursors entering the synthetic pathway downstream of the peroxisomal steps has been shown to rescue mitochondrial fragmentation.[77] Indeed, it has been demonstrated in plasmalogen deficient animal models that a modulation of the plasma plasmalogen levels can correct peripheral plasmalogen levels and rescue cardiac dysfunctions.[78] As such, targeting the Pls Score through dietary supplements holds great potential for individuals with conditions associated with peripheral plasmalogen deficiencies, including neurodegenerative diseases, cardiovascular disorders, and metabolic dysfunctions.

In conclusion, we devised a Pls Score that captures the relative circulating levels of PE and PE(P) and provided evidence supporting the link between the Pls Score and the risk of metabolic diseases, and all-cause mortality. Furthermore, we report associations of diet and lifestyle as well as the impact of SLO supplementation on the Pls Score. The observed associations between the Pls Score and metabolic diseases suggests its potential as a marker for metabolic health and to identify individuals at risk of metabolic diseases such as T2DM and CVD and premature death. The diet– Pls Score associations underscore the potential of targeted dietary and lifestyle interventions to effectively modify the Pls Score and improve metabolic health. Indeed, there is a need for a specialised clinical lipidomic platform[79,80] designed to measure the Pls Score within healthcare settings in order to offer a dependable method for evaluating metabolic health and risk, enabling well-informed clinical decision-making.

The prospective study design along with large sample size, and the inclusion of validation cohorts are the major strengths of the present study. However, there are also limitations: 1) The plasmalogen species measured are those that our current lipidomic platform can capture. Additional species, including those with low abundances, could also be useful; 2) Ethnicity of the present study populations was primarily white/European ancestry, which may limit the generalizability of the findings to

other populations. It is likely that normalisation of the Pls Score will be required for other ethnicities. Ethnicity can influence lipid metabolism and health outcomes, and it is possible that the associations observed with the Pls Score may vary across different ethnicities. 3) In the SLO study, we only investigated a single dose of the SLO mix. The effects of different doses and supplement regimens of SLO on plasmalogen levels and health outcomes warrant further exploration. Variations in dosage and treatment duration may have differential effects on plasmalogen metabolism and could influence the efficacy of SLO supplementation in mitigating metabolic risk factors. Furthermore, our investigation of SLO supplementation was constrained by a small sample size, which may limit the robustness of our conclusions and warrants cautious interpretation. Future research endeavours should address these limitations to enhance the validity and applicability of the Pls Score as a biomarker for metabolic health and guide the development of personalised interventions for diverse populations.

Contributors

HBB extracted plasma samples, performed LC-MS/MS analysis, analysed the data, and wrote the manuscript. SP extracted samples, performed LC-MS/MS analysis (SLO supplementation study). GO, CG, TW & TM provided statistical support and also reviewed the paper. CG and KH developed LC-MS/MS methods and provided support for the LC-MS/MS analysis and statistical analysis. MC developed extraction protocols and extracted plasma samples. NM supported the LC-MS/MS experiment and data pre-processing and analysis. GW, Jo.H, Je.H, J.Be, J.Bl and EM were involved in review & editing the manuscript. JES and DJM, coordinated the AusDiab data, interpreted results and revised the manuscript. PJM oversaw this work and revised the manuscript. PJM and CG are the guarantors of this work and shall take the responsibility for the full access and integrity of the data. All authors have approved the final version of the manuscript.

Data sharing statement

Because of the participant consent obtained as part of the recruitment process for the Australian Diabetes, Obesity and Lifestyle Study, it is not possible to make data publicly available (including the individual deidentified data). Individual-level data are available for analyses that do not conflict with ongoing studies, through application to the study lead Professor Jonathan Shaw and the AusDiab Study Committee (Email: Jonathan.Shaw@baker.edu.au). The timeframe for response to such requests is within two months.

Individual-level data for the Busselton Health Study are available under restricted access for analyses that do not conflict with ongoing studies; access is available through application to the Busselton Population Medical Research Institute (http://bpmri.org.au/research/database-access.html). Responses will be provided within 2 months.

The complete summary statistics for the Australian Diabetes, Obesity and Lifestyle Study and the Busselton Health Study are provided in the manuscript and Supplementary files. Source data are provided with this paper.

Declaration of interests

The Baker Institute has filed a provisional patent on the Plasmalogen Score. Application number: 2023900769; Title: Methods of assessing metabolic health. PJM consults for Juvenescence Ltd.

Acknowledgements

This research was supported by the National Health and Medical Research Council of Australia (Project grant 1101320) and Investigator grants to KH, JES, DJM, CG and PJM. This work was also supported in part by the Victorian Government's Operational Infrastructure Support Program. The AusDiab study, initiated and coordinated by the International Diabetes Institute, and subsequently coordinated by the Baker Heart and Diabetes Institute, gratefully acknowledges the support and assistance given by the AusDiab Study Team and all the study participants. Also, for funding or logistical support, we are grateful to: National Health and Medical Research Council (NHMRC grant 233200), Australian Government Department of Health and Ageing. Abbott Australasia Pty Ltd, Alphapharm Pty Ltd, AstraZeneca, Bristol-Myers Squibb, City Health Centre-Diabetes Service-Canberra, Department of Health and Community Services–Northern Territory, Department of Health and Human Services—Tasmania, Department of Health—New South Wales, Department of Health—Western Australia, Department of Health—South Australia, Department of Human Services—Victoria, Diabetes Australia, Diabetes Australia Northern Territory, Eli Lilly Australia, Estate of the Late Edward Wilson, GlaxoSmithKline, Jack Brockhoff Foundation, Janssen-Cilag, Kidney Health Australia, Marian & FH Flack Trust, Menzies Research Institute, Merck Sharp & Dohme, Novartis Pharmaceuticals, Novo Nordisk Pharmaceuticals, Pfizer Pty Ltd, Pratt Foundation, Queensland Health, Roche Diagnostics Australia, Royal Prince Alfred Hospital, Sydney, Sanofi Aventis, sanofi-synthelabo, and the Victorian Government's OIS Program. The authors wish to thank the staff at the Western Australian Data Linkage Branch and Death Registrations and Hospital Morbidity Data Collection for the provision of linked health data for the BHS. The SLO supplementation study was funded by the Baker Heart and Diabetes Institute seed fund grant scheme. The 1994/95 BHS was supported by a grant from the Health Promotion Foundation of Western Australia, and the authors acknowledge the generous support for the 1994/1995 BHS follow-up from Western Australia government and the Great Wine Estates of the Margaret River region of Western Australia and all the participants. Support from the Royal Perth Hospital Medical Research Foundation is also gratefully acknowledged. HBB was supported by the Baker institute and Monash University Scholarships. SP was supported by a Monash Graduate Scholarship and a Faculty Graduate Research International Scholarship from Monash University (SLO supplementation study). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105187.

References

1 Rapport MM, Lerner B, Alonzo N, Franzl RE. The structure of plasmalogens. Crystalline lysophosphatidal ethanolamine (acetal phospholipide). *J Biol Chem*. 1957;225(2):859–867.
2 Maeba R, Nishimukai M, Sakasegawa S-I, Sugimori D, Hara H. Advances in clinical chemistry. In: Makowski GS, ed. *Chapter two - plasma/serum plasmalogens: methods of analysis and clinical significance*. Elsevier; 2015:31–94.
3 Wallner S, Schmitz G. Plasmalogens the neglected regulatory and scavenging lipid species. *Chem Phys Lipids*. 2011;164(6):573–589.
4 Braverman NE, Moser AB. Functions of plasmalogen lipids in health and disease. *Biochim Biophys Acta*. 2012;1822(9):1442–1452.
5 Han X, Gross RW. Plasmenylcholine and phosphatidylcholine membrane bilayers possess distinct conformational motifs. *Biochemistry*. 1990;29(20):4992–4996.
6 Wallner S, Orsó E, Grandl M, Konovalova T, Liebisch G, Schmitz G. Phosphatidylcholine and phosphatidylethanolamine plasmalogens in lipid loaded human macrophages. *PLoS One*. 2018;13(10):e0205706.
7 Kuczynski B, Reo NV. Evidence that plasmalogen is protective against oxidative stress in the rat brain. *Neurochem Res*. 2006;31(5):639–656.
8 Jang JE, Park H-S, Yoo HJ, et al. Protective role of endogenous plasmalogens against hepatic steatosis and steatohepatitis in mice. *Hepatology*. 2017;66(2):416–431.
9 Zoeller RA, Lake AC, Nagan N, Gaposchkin DP, Legner MA, Lieberthal W. Plasmalogens as endogenous antioxidants: somatic

cell mutants reveal the importance of the vinyl ether. *Biochem J*. 1999;338:769–776.
10 da Silva TF, Eira J, Lopes AT, et al. Peripheral nervous system plasmalogens regulate Schwann cell differentiation and myelination. *J Clin Invest*. 2014;124(6):2560–2570.
11 Kurokin I, Lauer AA, Janitschke D, et al. Targeted lipidomics of mitochondria in a cellular Alzheimer's disease model. *Biomedicines*. 2021;9(8):1062.
12 Paul S, Lancaster GI, Meikle PJ. Plasmalogens: a potential therapeutic target for neurodegenerative and cardiometabolic disease. *Prog Lipid Res*. 2019;74:186–195.
13 Su XQ, Wang J, Sinclair AJ. Plasmalogens and Alzheimer's disease: a review. *Lipids Health Dis*. 2019;18(1):100.
14 Yamashita S, Kiko T, Fujiwara H, et al. Alterations in the levels of amyloid-β, phospholipid hydroperoxide, and plasmalogen in the blood of patients with Alzheimer's disease: possible interactions between amyloid-β and these lipids. *J Alzheimers Dis*. 2016;50:527–537.
15 Nishimukai M, Maeba R, Yamazaki Y, et al. Serum choline plasmalogens, particularly those with oleic acid in sn-2, are associated with proatherogenic state. *J Lipid Res*. 2014;55(5):956–965.
16 Mawatari S, Fukata M, Arita T, Maruyama T, Kono S, Fujino T. Decreases of ethanolamine plasmalogen and phosphatidylcholine in erythrocyte are a common phenomenon in Alzheimer's, Parkinson's, and coronary artery diseases. *Brain Res Bull*. 2022;189:5–10.
17 Hornburg D, Wu S, Moqri M, et al. Dynamic lipidome alterations associated with human health, disease and ageing. *Nat Metab*. 2023;5(9):1578–1594.
18 Wittenbecher C, Guasch-Ferré M, Haslam DE, et al. Changes in metabolomics profiles over ten years and subsequent risk of developing type 2 diabetes: results from the nurses' health study. *eBioMedicine*. 2022;75:103799.
19 Razquin C, Toledo E, Clish CB, et al. Plasma lipidomic profiling and risk of type 2 diabetes in the PREDIMED trial. *Diabetes Care*. 2018;41(12):2617–2624.
20 Fujino T, Hossain MS, Mawatari S. Therapeutic efficacy of plasmalogens for Alzheimer's disease, mild cognitive impairment, and Parkinson's disease in conjunction with a new hypothesis for the etiology of Alzheimer's disease. In: Lizard G, ed. *Peroxisome biology: experimental models, peroxisomal disorders and neurological diseases*. Cham: Springer International Publishing; 2020:195–212.
21 Goodenowe DB, Cook LL, Liu J, et al. Peripheral ethanolamine plasmalogen deficiency: a logical causative factor in Alzheimer's disease and dementia. *J Lipid Res*. 2007;48(11):2485–2498.
22 Rasmiena AA, Barlow CK, Stefanovic N, et al. Plasmalogen modulation attenuates atherosclerosis in ApoE- and ApoE/GPx1-deficient mice. *Atherosclerosis*. 2015;243(2):598–608.
23 Zhang M, Sun S, Tang N, Cai W, Qian L. Oral administration of alkylglycerols differentially modulates high-fat diet-induced obesity and insulin resistance in mice. *Evid Based Complement Alternat Med*. 2013;2013:834027.
24 Paul S, Smith AAT, Culham K, et al. Shark liver oil supplementation enriches endogenous plasmalogens and reduces markers of dyslipidemia and inflammation. *J Lipid Res*. 2021;62:100092.
25 Nguma E, Yamashita S, Kumagai K, et al. Ethanolamine plasmalogen suppresses apoptosis in human intestinal tract cells in vitro by attenuating induced inflammatory stress. *ACS Omega*. 2021;6(4):3140–3148.
26 Liu Y, Cong P, Zhang T, et al. Plasmalogen attenuates the development of hepatic steatosis and cognitive deficit through mechanism involving p75NTR inhibition. *Redox Biol*. 2021;43:102002.
27 Saitoh M, Itoh M, Takashima S, Mizuguchi M, Iwamori M. Phosphatidyl ethanolamine with increased polyunsaturated fatty acids in compensation for plasmalogen defect in the Zellweger syndrome brain. *Neurosci Lett*. 2009;449(3):164–167.
28 Dorninger F, Brodde A, Braverman NE, et al. Homeostasis of phospholipids - the level of phosphatidylethanolamine tightly adapts to changes in ethanolamine plasmalogens. *Biochim Biophys Acta*. 2015;1851(2):117–128.
29 Dunstan DW, Zimmet PZ, Welborn TA, et al. The Australian diabetes, obesity and lifestyle study (AusDiab)—methods and response rates. *Diabetes Res Clin Pract*. 2002;57(2):119–129.
30 Tapp RJ, Shaw JE, Harper CA, et al. The prevalence of and factors associated with diabetic retinopathy in the Australian population. *Diabetes Care*. 2003;26(6):1731–1737.
31 Gregory AT, Armstrong RM, Grassi TD, Gaut B, Van Der Weyden MB. On our selection: Australian longitudinal research studies. *Med J Aust*. 2008;189(11-12):650–657.
32 Cadby G, Melton PE, McCarthy NS, et al. Pleiotropy of cardiometabolic syndrome with obesity-related anthropometric traits determined using empirically derived kinships from the Busselton Health Study. *Hum Genet*. 2018;137(1):45–53.
33 Paul S, Rasmiena AA, Huynh K, et al. Oral supplementation of an alkylglycerol mix comprising different alkyl chains effectively modulates multiple endogenous plasmalogen species in mice. *Metabolites*. 2021;11(5):299.
34 Williams ED, Tapp RJ, Magliano DJ, Shaw JE, Zimmet PZ, Oldenburg BF. Health behaviours, socioeconomic status and diabetes incidence: the Australian diabetes obesity and lifestyle study (AusDiab). *Diabetologia*. 2010;53(12):2538–2545.
35 Briganti EM, McNeil JJ, Shaw JE, et al. Untreated hypertension among Australian adults: the 1999–2000 Australian diabetes, obesity and lifestyle study (AusDiab). *Med J Aust*. 2003;179(3):135–139.
36 Committee ADAPP. 2. Classification and diagnosis of diabetes: standards of medical care in diabetes—2022. *Diabetes Care*. 2021;45(1):17–38.
37 World Health Organization. *Definition, diagnosis and classification of diabetes mellitus and its complications; Part 1: diagnosis and classification of diabetes mellitus*. Geneva: Department of Noncommunicable Disease Surveillance, WHO; 1999.
38 Alshehry ZH, Barlow CK, Weir JM, Zhou Y, McConville MJ, Meikle PJ. An efficient single-phase method for the extraction of plasma lipids. *Metabolites*. 2015;5(2):389–403.
39 Huynh K, Barlow CK, Jayawardana KS, et al. High-throughput plasma lipidomics: detailed mapping of the associations with cardiometabolic risk factors. *Cell Chem Biol*. 2019;26(1):71–84.
40 Kevin Huynh Natalie AM, Duong T, Van Anh NT, Meikle TG, Giles C, Meikle PJ. In: Baker Heart and Diabetes Institute Melbourne A, ed. *A comprehensive, curated, high-throughput method for the detailed analysis of the plasma lipidome*. Agilent Technologies, Inc; 2021.
41 A comprehensive, curated, high-throughput method for the detailed analysis of the plasma lipidome. https://metabolomics.baker.edu.au/method/; 2023.
42 Cadby G, Melton PE, McCarthy NS, et al. Heritability of 596 lipid species and genetic correlation with cardiovascular traits in the Busselton family heart study. *J Lipid Res*. 2020;61(4):537–545.
43 Webb-Robertson BJ, Matzke MM, Jacobs JM, Pounds JG, Waters KM. A statistical selection strategy for normalization procedures in LC-MS proteomics experiments through dataset-dependent ranking of normalization scaling factors. *Proteomics*. 2011;11(24):4736–4741.
44 Meikle PJ, Wong G, Barlow CK, et al. Plasma lipid profiling shows similar associations with prediabetes and type 2 diabetes. *PLoS One*. 2013;8(9):e74341.
45 Prada M, Eichelmann F, Wittenbecher C, Kuxhaus O, Schulze MB. Plasma lipidomic n-6 polyunsaturated fatty acids and type 2 diabetes risk in the EPIC-potsdam prospective cohort study. *Diabetes Care*. 2023;46(4):836–844.
46 Eichelmann F, Sellem L, Wittenbecher C, et al. Deep lipidomics in human plasma: cardiometabolic disease risk and effect of dietary fat modulation. *Circulation*. 2022;146(1):21–35.
47 Lu J, Lam SM, Wan Q, et al. High-Coverage targeted lipidomics reveals novel serum lipid predictors and lipid pathway dysregulation antecedent to type 2 diabetes onset in normoglycemic Chinese adults. *Diabetes Care*. 2019;42(11):2117–2126.
48 Arita T, Yokoyama T, Moriyama S, et al. Plasma and erythrocyte membrane plasmalogens in patients with coronary heart diseases undergoing percutaneous intervention. *Cardiol Angiology*. 2018;7(4):1–11.
49 Huynh K, Lim WLF, Giles C, et al. Concordant peripheral lipidome signatures in two large clinical studies of Alzheimer's disease. *Nat Commun*. 2020;11(1):5698.
50 Kling MA, Goodenowe DB, Senanayake V, et al. Circulating ethanolamine plasmalogen indices in Alzheimer's disease: relation to diagnosis, cognition, and CSF tau. *Alzheimers Dement*. 2020;16(9):1234–1247.
51 Maeba R, Maeda T, Kinoshita M, et al. Plasmalogens in human serum positively correlate with high- density lipoprotein and decrease with aging. *J Atherosclerosis Thromb*. 2007;14(1):12–18.
52 Maeba R, Kojima K-I, Nagura M, et al. Association of cholesterol efflux capacity with plasmalogen levels of high-density lipoprotein: a cross-sectional study in chronic kidney disease patients. *Atherosclerosis*. 2018;270:102–109.

53 Honsho M, Abe Y, Fujiki Y. Dysregulation of plasmalogen homeostasis impairs cholesterol biosynthesis. *J Biol Chem*. 2015;290(48):28822–28833.
54 Beyene HB, Olshansky G, AA TS, et al. High-coverage plasma lipidomics reveals novel sex-specific lipidomic fingerprints of age and BMI: evidence from two large population cohort studies. *PLoS Biol*. 2020;18(9):e3000870.
55 Titorenko VI, Terlecky SR. Peroxisome metabolism and cellular aging. *Traffic*. 2011;12(3):252–259.
56 Pradas I, Jové M, Huynh K, et al. Exceptional human longevity is associated with a specific plasma phenotype of ether lipids. *Redox Biol*. 2019;21:101127.
57 Dean JM, Lodhi IJ. Structural and functional roles of ether lipids. *Protein Cell*. 2018;9(2):196–206.
58 Meikle PJ, Wong G, Tsorotes D, et al. Plasma lipidomic analysis of stable and unstable coronary artery disease. *Arterioscler Thromb Vasc Biol*. 2011;31(11):2723–2732.
59 Stegemann C, Pechlaner R, Willeit P, et al. Lipidomics profiling and risk of cardiovascular disease in the prospective population-based bruneck study. *Circulation*. 2014;129(18):1821–1831.
60 Harris WS. Omega-3 fatty acids and cardiovascular disease: a case for omega-3 index as a new risk factor. *Pharmacol Res*. 2007;55(3):217–223.
61 Harris WS, Del Gobbo L, Tintle NL. The Omega-3 Index and relative risk for coronary heart disease mortality: estimation from 10 cohort studies. *Atherosclerosis*. 2017;262:51–54.
62 Jové M, Naudí A, Gambini J, et al. A stress-resistant lipidomic signature confers extreme longevity to humans. *J Gerontol A Biol Sci Med Sci*. 2017;72(1):30–37.
63 Wang F, Tessier A-J, Liang L, et al. Plasma metabolomic profiles associated with mortality and longevity in a prospective analysis of 13,512 individuals. *Nat Commun*. 2023;14(1):5744.
64 Gonzalez-Covarrubias V, Beekman M, Uh HW, et al. Lipidomics of familial longevity. *Aging Cell*. 2013;12(3):426–434.
65 Wu Y, Chen Z, Jia J, Chiba H, Hui SP. Quantitative and comparative investigation of plasmalogen species in daily foodstuffs. *Foods*. 2021;10(1):124.
66 Yamashita S, Kanno S, Honjo A, et al. Analysis of plasmalogen species in foodstuffs. *Lipids*. 2016;51(2):199–210.
67 Boselli E, Pacetti D, Lucci P, Frega NG. Characterization of phospholipid molecular species in the edible parts of bony fish and shellfish. *J Agric Food Chem*. 2012;60(12):3234–3245.
68 Wood PL, Muir W, Christmann U, et al. Lipidomics of the chicken egg yolk: high-resolution mass spectrometric characterization of nutritional lipid families. *Poultry Sci*. 2021;100(2):887–899.
69 Mason RP, Libby P, Bhatt DL. Emerging mechanisms of cardiovascular protection for the omega-3 fatty acid eicosapentaenoic acid. *Arterioscler Thromb Vasc Biol*. 2020;40(5):1135–1147.
70 Wang-Sattler R, Yu Y, Mittelstrass K, et al. Metabolic profiling reveals distinct variations linked to nicotine consumption in humans — first results from the KORA study. *PLoS One*. 2008;3(12):e3863.
71 Lietz M, Berges A, Lebrun S, et al. Cigarette-smoke-induced atherogenic lipid profiles in plasma and vascular tissue of apolipoprotein E-deficient mice are attenuated by smoking cessation. *Atherosclerosis*. 2013;229(1):86–93.
72 Rogerson MC, Le Grande MR, Dunstan DW, et al. Television viewing time and 13-year mortality in adults with cardiovascular disease: data from the Australian diabetes, obesity and lifestyle study (AusDiab). *Heart Lung Circ*. 2016;25(8):829–836.
73 Dunstan DW, Barr EL, Healy GN, et al. Television viewing time and mortality: the Australian diabetes, obesity and lifestyle study (AusDiab). *Circulation*. 2010;121(3):384–391.
74 Grøntved A, Hu FB. Television viewing and risk of type 2 diabetes, cardiovascular disease, and all-cause mortality: a meta-analysis. *JAMA*. 2011;305(23):2448–2455.
75 Brites P, Ferreira AS, da Silva TF, et al. Alkyl-glycerol rescues plasmalogen levels and pathology of ether-phospholipid deficient mice. *PLoS One*. 2011;6(12):e28539.
76 Deniau A-L, Mosset P, Le Bot D, Legrand AB. Which alkylglycerols from shark liver oil have anti-tumour activities? *Biochimie*. 2011;93(1):1–3.
77 Hu D, Tan M, Lu D, et al. TMEM135 links peroxisomes to the regulation of brown fat mitochondrial fission and energy homeostasis. *Nat Commun*. 2023;14(1):6099.
78 Todt H, Dorninger F, Rothauer PJ, et al. Oral batyl alcohol supplementation rescues decreased cardiac conduction in ether phospholipid-deficient mice. *J Inherit Metab Dis*. 2020;43(5):1046–1055.
79 Meikle TG, Huynh K, Giles C, Meikle PJ. Clinical lipidomics: realizing the potential of lipid profiling. *J Lipid Res*. 2021;62:100127.
80 Vvedenskaya O, Holčapek M, Vogeser M, Ekroos K, Meikle PJ, Bendt AK. Clinical lipidomics – a community-driven roadmap to translate research into clinical applications. *J Mass Spectrom Adv Clin Lab*. 2022;24:1–4.