Mid-life anti-inflammatory metabolites are inversely associated with long-term cardiovascular disease events

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Summary

Background Preclinical data have shown that low levels of metabolites with anti-inflammatory properties may impact metabolic disease processes. However, the association between mid-life levels of such metabolites and long-term ASCVD risk is not known.

Methods We characterised the plasma metabolomic profile (1228 metabolites) of 1852 participants (58.1 ± 7.5 years old, 69.6% female, 43.6% self-identified as Black) enrolled in the Heart Strategies Concentrating on Risk Evaluation (Heart SCORE) study. Logistic regression was used to assess the impact of metabolite levels on ASCVD risk (nonfatal MI, revascularisation, and cardiac mortality). We additionally explored the effect of genetic variants neighbouring ASCVD-related genes on the levels of metabolites predictive of ASCVD events. The Atherosclerosis Risk in Communities (ARIC) study (n = 4790; 75.5 ± 5.1 years old, 57.4% female, 19.5% self-identified as Black) was used as an independent validation cohort.

Findings In fully adjusted models, alpha-ketobutyrate [AKB] (OR 0.62 [95% CI, 0.49–0.80]; p < 0.001), and 1-palmitoyl-2-linoleoyl-GPI [OR, 0.62, 95% CI, 0.47–0.83; p < 0.001], two metabolites in amino acid and phosphatidylinositol lipid pathways, respectively, showed a significant protective association with incident ASCVD risk in both Heart SCORE and ARIC cohorts. Three plasmalogens and a bilirubin derivative, whose levels were regulated by genetic variants neighbouring FADS1 and UGT1A1, respectively, exhibited a significant protective association with ASCVD risk in the Heart SCORE only.

Interpretation Higher mid-life levels of AKB and 1-palmitoyl-2-linoleoyl-GPI metabolites may be associated with lower risk late-life ASCVD events. Further research can determine the causality and therapeutic potential of these metabolites in ASCVD.

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Keywords: Atherosclerosis; ASCVD prevention; Alpha-ketobutyrate; Metabolomics

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Research in context

Evidence before this study

- The role of inflammation and oxidative stress in atherosclerotic cardiovascular disease (ASCVD) development is established
- Plasmalogens and 1-palmitoyl-2-linoleoyl-GPI, linked to anti-inflammatory and antioxidant effects, have shown potential in cardiovascular health, but longitudinal evidence and genetic links, such as FADS1/FADS2, are less known
- Preclinical studies have demonstrated that α-ketobutyrate can extend lifespan and in *Caenorhabditis elegans* however, human data are lacking.

Added value of this study

- Mid-life levels of alpha-ketobutyrate and phosphatidylinositol metabolites are strong markers of ASCVD risk later in life.
- Plasmalogens may help prevent ASCVD in mid-life but appear less protective in older individuals. Genetic associations with FADS1/FADS2 offer mechanistic insights into their cardioprotective effects.
- Levels of these validated metabolites with anti-inflammatory effects may be regulated by environmental factors such as diet

Implications of all the available evidence

- Early identification of anti-inflammatory metabolites may enhance ASCVD risk stratification for primordial and primary prevention of ASCVD.
- Future studies should explore causality and validate the use of these metabolites for targeted ASCVD prevention strategies

Introduction

Atherosclerotic cardiovascular disease (ASCVD) is a leading cause of mortality and morbidity worldwide.[1] The process of atherosclerosis begins decades before clinical manifestations of ASCVD events.[2,3] Therefore, identification of pathophysiologic and protective markers of atherosclerosis in mid-life is essential in primordial and primary prevention of ASCVD events in late-life.[4,5] These markers can also provide insights into the development of unique targets for developing preventative therapies.

It is well known that inflammation-induced oxidative stress,[6,7] dysregulated lipid metabolism[8,9] and elevated remnant cholesterol[10–12] contribute to the residual risk of cardiovascular disease (CVD). This residual risk is beyond what is captured by traditional lipid profiles[11] and clinically available risk assessment tools. The use of metabolomic analyses for comprehensive profiling of molecular markers in the lipid, amino acid and other pathways in systemic disease processes[13–15] provides an opportunity to identify the role of these metabolites in the development of ASCVD.

Preclinical data indicate that low levels of plasmalogens, a class of membrane glycerophospholipids, modulate cardiometabolic changes[16] which could lead to atherosclerosis. However, population level associations of mid-life plasmalogens and other metabolites with anti-inflammatory properties with incident ASCVD events are lacking. Here, we assessed midlife metabolomic architecture with risk of incident ASCVD events over a longitudinal follow-up period in the Heart Strategies Concentrating on Risk Evaluation (Heart SCORE) study. Given the known genetic influences on ASCVD associated markers,[17,18] we then investigated genetic level associations with metabolites found to be related with incident ASCVD events. The Atherosclerosis Risk in Communities (ARIC) study was utilised for validation of our findings in the Heart SCORE study.

Methods

Study population

Heart SCORE study

The Heart Strategies Concentrating on Risk Evaluation (Heart SCORE) study[19,20] began in 2003 as a community-based participatory research study of race-related disparities in ASCVD conducted in Allegheny County, Pennsylvania. A detailed description of the Heart SCORE study design has been published elsewhere.[19,20] The study enrolled 2000 participants age 45–75 years. Those with a life expectancy <5 years or an inability to undergo annual follow-up visits were excluded.

ARIC study (Validation Cohort)

The Atherosclerosis Risk in Communities (ARIC) study is a prospective community-based study of ASCVD incidence in 15,792 middle-aged (age 45–64 years at visit 1) adults recruited from 4 U.S. communities from 1987 to 1989. A detailed description of the ARIC study design and methods has been published elsewhere.[21,22] Validation analysis for this study was conducted among individuals who participated in ARIC visit 5 (age 69–88 years), conducted between June 1, 2011, and August 30, 2013. The follow up period of ARIC study was over a mean of 7.5 years.

Assessment of covariates

Baseline evaluation included assessment of demographics, psychosocial characteristics, anthropometric measurements, exercise and dietary habits, traditional ASCVD risk factors, and sleep disturbances. Diabetes mellitus was defined as a fasting serum

glucose level ≥126 mg/dL or non-fasting serum glucose level ≥200 mg/dL, a self-reported physician diagnosis of diabetes, or use of hypoglycaemic medication. Participants were classified as "never," "former," or "current" smokers from self-reported information. Blood pressure (BP) was measured by trained technicians following a standard protocol. Fasting venous lipid profile was measured using standard enzymatic methods[23] at the University of Pittsburgh Medical Center clinical laboratory. Interleukin (IL-6) levels were measured using commercially available ELISA kits (Quantikine high-sensitivity [hs] human kits, R&D Systems, Minneapolis, Minn). High-sensitivity C-reactive protein (hs-CRP) was measured by a high-sensitivity method on a Hitachi 911 analyser (Roche Diagnostics, Basel, Switzerland) using reagents from Denka Seiken (Niigata, Japan). Baseline plasma samples were stored for future analyses. The present analyses were confined to 1852 participants who self-reported sex (as man or woman) and race (as either Black or White).

Metabolite profiling

Metabolomic profiling was completed on plasma from samples obtained in the fasting state at the baseline visit and stored at −80 °C. Duplicate technical replicates were done for 99 participants for validation purposes, where one replicate was chosen at random to be used in all downstream analyses. Equal volumes of all samples were extracted and run across the Metabolon Inc. (Durham, NC) Precision Metabolomics discovery platform.[24,25] Samples were extracted and split into equal parts for analysis on four LC/MS/MS platforms. Proprietary software was used to match ions to an in-house library of standards for metabolite identification and for metabolite quantitation by peak area integration, where a metabolite's abundance was defined as its peak area. Metabolites with >10% missing data were discarded to avoid missing data-related biases. Remaining missing data was imputed as the metabolite's minimum observed abundance. As we have done previously,[26] we used the Metabolomics Standard Initiative to classify metabolites as annotated (level 1 or 2; identified or putatively annotated compounds), semi-annotated (level 3; putatively characterised compound classes), and non-annotated (level 4; unknown compounds).[27]

Genetic profiling

Blood samples for genotyping were collected in 10 mmol/L EDTA. DNA was isolated following standard protocols. A customised Illumina CARe iSelect (IBC) cardiovascular array developed for cardiometabolic phenotypes[28] was used for genotyping. The IBC array is a 50 K SNP chip that assays polymorphisms in 2100 genes.

Incident cardiovascular disease events

Ongoing annual evaluations include measurement of risk factors, tabulation and adjudication of adverse events in the Heart SCORE study. The primary outcome was a composite of ASCVD mortality and nonfatal ASCVD events, defined as nonfatal myocardial infarction (MI), acute ischaemic syndrome, coronary revascularisation (percutaneous coronary intervention or coronary artery bypass graft). All events were tabulated by annual telephonic or in-person follow up, confirmed by hospital record review, and adjudicated by staff cardiologists. Mortality was ascertained and classified as ASCVD death by reviewing death certificates and hospital records. Outcomes were analysed until December 31, 2020 with a median of 12.1 (9.2, 12.3) years follow up period.

For validation in the ARIC study, a composite endpoint of adjudicated incident ASCVD events included incident MI hospitalisation, non-fatal MI, revascularisation, and cardiac mortality. While the events of interest were documented and adjudicated according to each of the study's individual protocol, this composite endpoint was consistently applied across both studies to facilitate meaningful comparison of outcomes.

Statistical analyses

Baseline comparisons

Baseline characteristics between Black and White participants are compared using the Welch Two Sample t-test and Pearson's Chi-squared tests for continuous and categorical variables, respectively. To assess the presence of unequal variances formally, we applied Levene's test for homogeneity of variances prior to conducting the Welch t-test.

Identification of Metabolite-ASCVD associations in Heart SCORE Study

Latent factors that might confound the relationship between metabolite levels and ASCVD events were estimated using the method described by McKennan et al.[29] For each metabolite, we used logistic regression to regress ASCVD (yes/no) onto that metabolite's log-abundance while controlling for latent factors, the baseline covariates included in the Pooled Cohorts Equations (PCE) (age, sex, self-reported race, hypertension medication use, systolic BP, smoking status, diabetes mellitus, high density lipoprotein (HDL) cholesterol, total cholesterol),[30,31] and the log-concentrations of the inflammatory markers hs-CRP and IL-6. The Benjamini-Hochberg procedure[32] was used to control the false discovery rate (FDR) at 10%. ASCVD odds ratios were calculated as the change in odds after increasing a metabolite's log-abundance by one standard deviation.

Validation in ARIC Study

An identical analysis to the one above was utilised for latent factor estimation and analysis (19, 24) in the ARIC study. Since nine of the twelve ASCVD-related metabolites identified in Heart SCORE were also available in ARIC, the Bonferroni p-value threshold 0.05/9 was used

to validate significant metabolites identified in Heart SCORE.

Risk prediction of ASCVD

To assess the utility of the significant metabolites for risk stratification and prediction, we used statistical measures of discrimination including the area under the receiver operating characteristic curve (AUC)[33] and net reclassification index (NRI)[34] to calculate the incremental value of adding metabolites to the currently applicable risk prediction models. We then tested the performance of a *base model* (PCE variables with and without inflammatory markers) with candidate metabolites that were validated (*2M model*) and then all metabolites identified in Heart SCORE (*allM model*). To avoid over-fitting, we use a leave-one-out paradigm to derive receiver operator curves (ROC) curves. Briefly, for each sample, we leave that sample out of the dataset and use elastic net to fit a risk model for ASCVD events and subsequently predict ASCVD events for the held-out sample. The p-values are for the null hypothesis that the difference between the area under the ROC curves (AUCs) for a model and the baseline model is zero.

Metabolite Genome-Wide Association Study (GWAS)

DNA samples from 1132 Heart SCORE participants with metabolite and baseline covariate information were used to perform a metabolite GWAS. Quality control (QC) was performed by removing SNPs with >10% missing data, with minor allele frequency <1%, or in Hardy–Weinberg disequilibrium (p-value $\leq 10^{-5}$) in either self-identified whites or blacks, which resulted in 43,465 candidate SNPs. The method of Zhao et al.[35] was used to perform two independent metabolomic GWAS's, which estimate indirect and direct genetic effects on metabolite levels where indirect effects are mediated through latent factors and the direct effect analysis is equivalent to standard latent factor-corrected quantitative trait loci analyses.[36] Adjustments were made for baseline covariates and percent African ancestry in each GWAS. Metabolite-SNP or ASCVD-metabolite-SNP pairs with p-values $\leq 0.05/(\#\text{Metabolites}*\#\text{SNPs})$ or $\leq 0.05/(\#\text{ASCVD-metabolites}*\#\text{SNPs})$ from either GWAS were considered metabolome-wide or ASCVD-metabolite-wide significant, respectively. Metabolome-wide significance refers to adjusting the total number of tests (T = #metabolites x #SNPs) in the metabolite GWAS. ASCVD-metabolites are metabolites that were significantly related to ASCVD in Heart SCORE (see above).

Ethics

All participants in the HeartSCORE study provided written informed consent approved by the University of Pittsburgh Institutional Review Board (IRB). The ARIC study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki, and all procedures were approved by the IRBs at each of the four study sites (University of North Carolina at Chapel Hill, University of Minnesota, Johns Hopkins University, and University of Mississippi Medical Center). All participants provided written informed consent at the time of enrolment, and their confidentiality and privacy were rigorously protected.

Role of funders

The funders had no role in study design, data collection, data analyses, interpretation, or writing of report. The funders specifically disclaim responsibility for any analyses, interpretations, or conclusions.

Results

The 1852 participants were 59.1 ± 7.5 years of age at study entry (Table 1) in the Heart SCORE study. Women comprised 65.8% of the cohort; 43.6% of participants self-identified as Black. Black participants had a higher prevalence of high Framingham risk (25.1 vs 13.7%), were more likely to be smokers (14.4 vs 8%), and had higher prevalence of hypertension (55.9 vs 38.3%) and diabetes (15.8 vs 5.3%) as compared to White counterparts. White participants had nominally higher total cholesterol levels (216 vs 209 mg/dL). Additionally stratified baseline characteristics by ASCVD events are noted in Supplemental Table i.

The composite endpoint of incident ASCVD events occurred in 7.5% of participants followed for median of 12.1 (9.2, 12.3) years. This included ASCVD mortality (2.9%) and incident MI (2.6%). Cardiac mortality was noted to be higher in Black compared to White participants (3.8% vs 2.2%, respectively; p-value = 0.04).

A total of 4315 ARIC study participants were included in the replication cohort with mean age 75.5 ± 5.1 years (56.9% women and 17.8% self-identified as Black) (Table 2). The composite endpoint of incident ASCVD events occurred in 9.9% of participants followed over a mean of 7.5 years.

Distribution of metabolites in heart SCORE

A condensed Heat map of the Heart SCORE cohort metabolite distribution with both self-reported sex and racial comparisons is included as Supplemental Figure ii. In both Black and White participants, males had higher levels of amino acid-associated metabolites and lower levels of many lipid species, except for acyl carnitines and steroids. Plasmalogen levels were nominally higher in Black participants at baseline compared to White participants.

Identification of metabolites associated with incident ASCVD events

Of the 1228 metabolites quantified in Heart SCORE participants, eight annotated, one semi-annotated, and

	Overall (N = 1852)	Black (N = 808)	White (N = 1044)	p-value
Age, y	59.1 (7.5)	58.1 (7.5)	59.8 (7.4)	<0.001
Female	1218 (65.8%)	562 (69.6%)	656 (62.8%)	0.003
BMI, Mean (SD)	30.1 (6.2)	32.0 (6.4)	28.6 (5.5)	<0.001
Hypertension	852 (46.0%)	452 (55.9%)	400 (38.3%)	<0.001
Systolic BP (mmHg)	137 (19.8)	141 (20.1)	133 (18.8)	<0.001
Current smoking	200 (10.8%)	116 (14.4%)	84 (8.0%)	<0.001
Diabetes mellitus	183 (9.9%)	128 (15.8%)	55 (5.3%)	<0.001
Total cholesterol, mg/dL	213 (42.7)	209 (44.4)	216 (41.1)	<0.001
HDL-C, mg/dL	57.6 (14.9)	58.1 (14.4)	57.2 (15.3)	0.2
Hs-CRP mg/dL	0.363 (1.2)	0.63 (1.2)	0.16 (1.2)	<0.001
IL-6 pg/mL	0.525 (0.75)	0.78 (0.68)	0.36 (0.75)	<0.001

Data are displayed as mean ± SD for continuous variables and n (%) for categorical variables. Abbreviations: SD, standard deviation; BMI, body mass index; BP, blood pressure; HDL-C, high-density lipoprotein cholesterol; HS-CRP, high sensitivity C reactive protein, IL-6, Interleukin-6.

Table 1: **Baseline characteristics stratified by self-reported race in Heart SCORE study.**

three non-annotated metabolites were significantly associated with incident ASCVD events (Fig. 1). Of the eight annotated metabolites, six were lipids (three plasmalogens, one phosphatidylinositol [PI], one phosphatidylethanolamine, and one long chain fatty acid) and two were from the amino acid pathway (alpha-ketobutyrate and 2-oxarginine). The semi-annotated metabolite, whose Metabolon-assigned ID was X-16946, had the chemical formula $C_{16}H_{18}N_2O_5$ and is likely a bilirubin degradation product.[37] Eleven out of the 12 significant metabolites exhibited protective associations with ASCVD risk (Fig. 2a). Survival analysis assessments yielded similar results (Supplemental Table ii).

All three plasmalogens, alpha-ketobutyrate, and the PI metabolite (1-palmitoyl-2-linoleoyl-GPI) were associated with dietary intake of animal protein and dark green leafy vegetables (Supplement Tables iii and iva and b). The levels of all three plasmalogens were additionally positively correlated with HDL cholesterol levels (p-values <0.001).

Validation of metabolites in ARIC study

As shown in Fig. 2b, both alpha-ketobutyrate (OR 0.62, 95% CI [0.65, 0.87]; p < 0.001) and the PI metabolite 1-palmitoyl-2-linoleoyl-GPI OR 0.86, 95% CI [0.78, 0.94]; p = 0.017) showed a significantly inverse association with incident ASCVD events in the ARIC study cohort after adjusting for multiple testing. Table 3 summarises OR intervals in Heart SCORE and ARIC for all ASCVD-related metabolites.

While this provides substantial evidence that the ASCVD-related metabolites we identified in Heart SCORE replicate in other populations, we hypothesised that some metabolites could not be validated in ARIC because ARIC study participants were generally older than Heart SCORE's at the time of metabolite sampling (75 y vs 59 y; Tables 1 and 2). We tested this

	Total[a] (N = 4734)	Black[a] (N = 897)	White[a] (N = 3837)	p-value
Age (years)	75.4 (5.08)	74.7 (5.01)	75.6 (5.08)	<0.001
Female	2697 (56.9%)	581 (64.7%)	2116 (55.2%)	<0.001
BMI	28.7 (5.56)	30.6 (6.70)	28.20 (5.16)	<0.001
Systolic BP (mmHg)	129.93 (17.99)	134.08 (19.26)	128.96 (17.54)	<0.001
Total cholesterol (mmol/L)	4.68 (1.07)	4.70 (1.01)	4.67 (1.09)	0.313
HDL-C (mmol/L)	1.35 (0.360)	1.37 (0.346)	1.34 (0.365)	0.0056
Current smoking	275 (5.8%)	57 (6.4%)	218 (5.7%)	0.486
Prevalent Diabetes	1521 (32.1%)	393 (43.81%)	1128 (29.4%)	<0.001
Anti-Hypertensive use	3140 (66.3%)	739 (82.4%)	2401 (62.6%)	<0.001
Hs-CRP (RFU)	14.2 (1.08)	14.4 (1.11)	14.1 (1.07)	<0.001
IL-6 (RFU)	8.19 (0.503)	8.22 (0.519)	8.19 (0.499)	0.072

Data are displayed as mean ± SD for continuous variables and n (%) for categorical variables. Abbreviations: SD, standard deviation; BMI, body mass index; BP, blood pressure; HDL-C, high-density lipoprotein cholesterol; HS-CRP, high sensitivity C reactive protein, IL-6, Interleukin-6. [a]n (%); Mean (SD).

Table 2: **Baseline characteristics stratified by self-reported race in ARIC study, visit 5.**

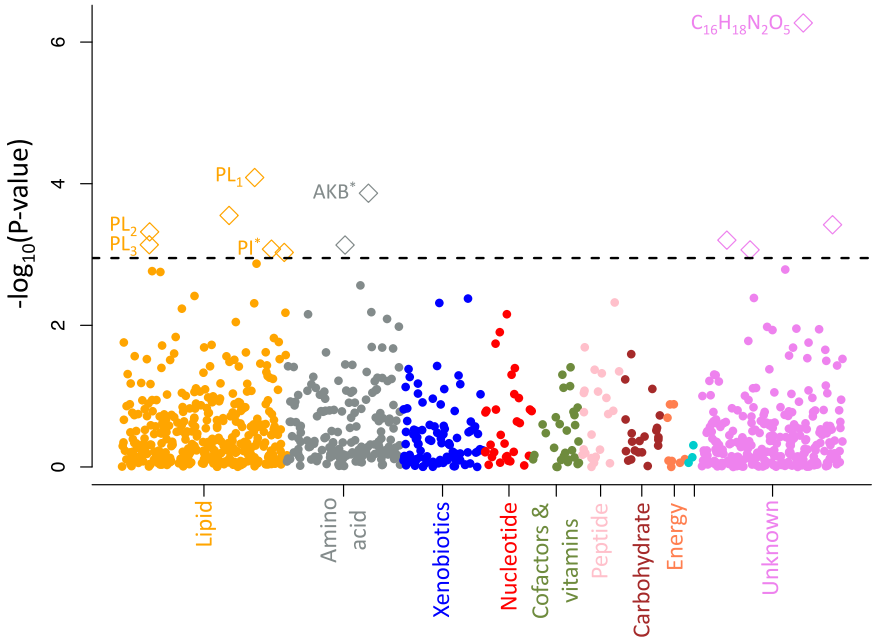

Fig. 1: Metabolomic Pathways and Incident Atherosclerotic Cardiovascular Disease (ASCVD) Events in the Heart SCORE Study. This figure illustrates the metabolomic pathways significantly associated with incident ASCVD events in the Heart SCORE study. Each pathway's contribution to ASCVD risk is highlighted, along with the key metabolites driving the associations.

by adding a metabolite by age (≥65 y or <65 y) interaction term in our Heart SCORE regression models, which allowed us to estimate the difference in the effect of metabolite levels on ASCVD risk in older (≥65 y) and younger (<65 y) subjects. Notably, we found that the effect of all three ASCVD-related plasmalogens was significantly dampened in older subjects [Supplemental Table v], suggesting plasmalogens could not be replicated because their levels in the older ARIC participants have little-to-no impact on ASCVD risk.

Risk prediction of ASCVD

For risk prediction of ASCVD, the base model, which included all non-metabolite predictors of ASCVD included in the PCE, presented fair discrimination (AUC = 0.713). Evaluation for the incremental benefit of adding the two validated metabolites (alpha-ketobutyrate and 1-palmitoyl-2-linoleoyl-GPI) showed a small but significantly improved discrimination (ΔAUC 0.024, p-value = 0.011) [Supplemental Figure iii; yellow curve]. Adding all significant Heart SCORE metabolites (model allM) showed a robust improvement when added to the base model (**ΔAUC 0.093**, p-value<0.001) [Supplemental Figure iv] and NRI index (Supplemental Table iva). When significant metabolites were added to only the PCE variables (without inflammatory biomarkers); similarly significant increments were noted in the risk prediction indices (Supplemental Figure iv, Supplemental Table ivb).

Metabolite genome-wide association study

We identified 641 metabolome-wide significant metabolite-SNP pairs, comprising 120 metabolites from 42 pathways and 193 SNPs from 74 genes. Notably, the abundances of two out of the three significant ASCVD-related Plasmalogens were associated with the genotype at rs174535 in FADS1/2 (Fig. 3a and b). This SNP is in near perfect linkage equilibrium with both *rs174547* and *rs174546* (r2 = 0.98 in Europeans), whose genotypes have been shown to modify aortic stenosis[38] and coronary artery disease risk,[39] respectively. We also found the ASCVD-related metabolite $C_{16}H_{18}N_2O_5$ to be strongly associated with SNPs on UGT1A (Fig. 3a), a regulator of bilirubin metabolism[40] which is consistent with $C_{16}H_{18}N_2O_5$ being a bilirubin degradation product.

Discussion

We identified that mid-life levels of unique plasma metabolites, with anti-inflammatory and anti-oxidant properties, are inversely associated with incident ASCVD events in late-life. Specifically, our data indicate that higher levels of both alpha-ketobutyrate, an amino acid metabolite, and 1-palmitoyl-2-linoleoyl-GPI, a phosphatidylinositol (PI) lipid, are protective for ASCVD events in both the Heart SCORE and the ARIC study over a decade long follow up period. Both these metabolites were also shown to nominally improve longitudinal ASCVD risk prediction (Supplemental Figure ii). Furthermore, within just the Heart

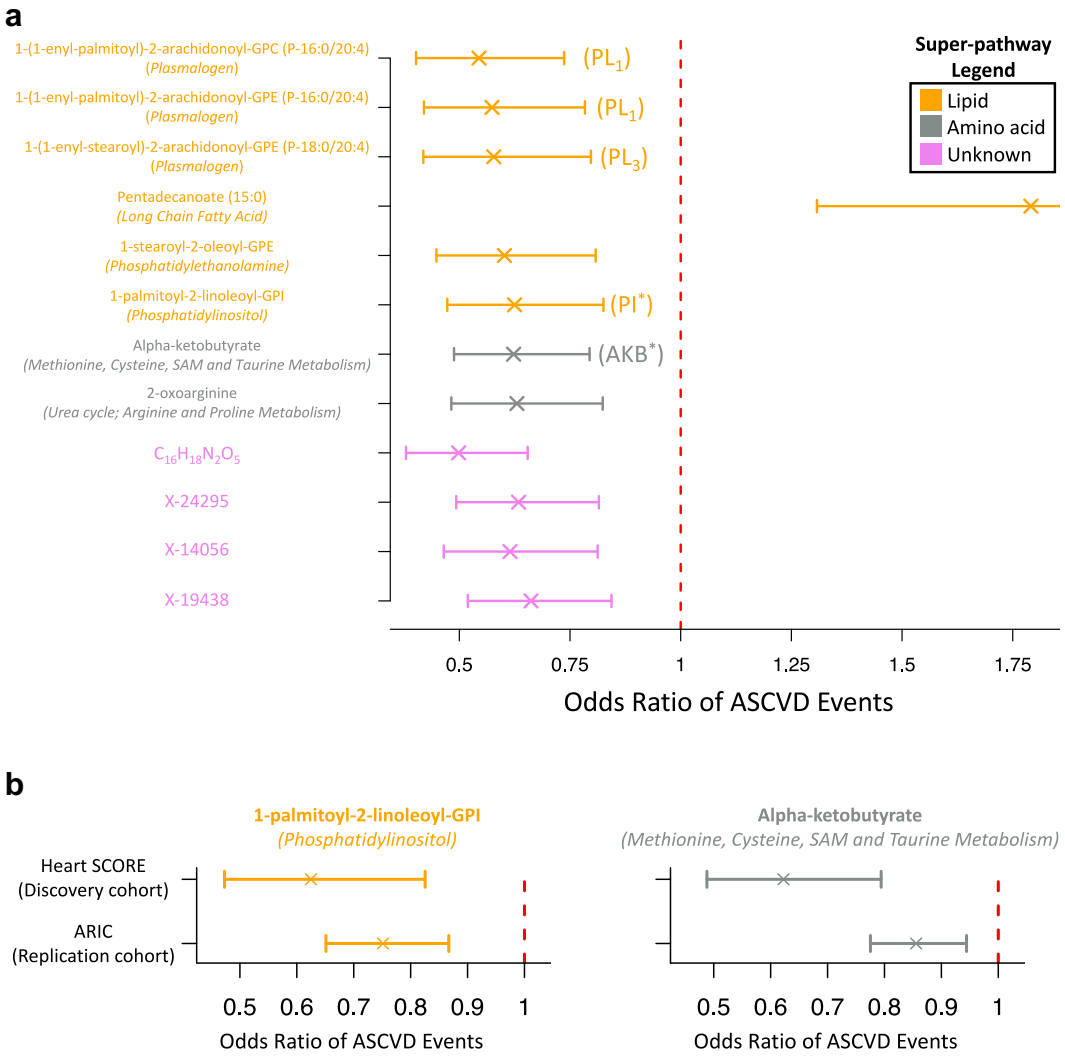

Fig. 2: **a**: Significant metabolites and ASCVD Risk in the Heart SCORE Study. This panel displays the associations between individual significant metabolites identified in the Heart SCORE study and ASCVD risk. Hazard ratios with confidence intervals are presented to demonstrate the strength and direction of associations. **b:** Validation of Significant Metabolites in the ARIC Study. This panel confirms the external validity of significant metabolites identified in the Heart SCORE study by replicating the findings in the ARIC study. Results include hazard ratios and confidence intervals, validating the predictive utility of these metabolites across cohorts.

SCORE study, three *arachidonoyl* plasmalogens from the lipid pathway; (1-(1-enyl-palmitoyl)-2-arachidonoyl-GPC, 1-(1-enyl-palmitoyl)-2-arachidonoyl-GPE and 1-(1-enyl-stearoyl)-2-arachidonoyl-GPE) are inversely associated with ASCVD risk over a median of 12.1 years of follow-up, independent of race, traditional ASCVD risk factors and inflammatory markers. Metabolome-wide genetic analysis revealed that two of these Plasmalogens are strongly influenced by polymorphisms on the FADS1/FADS2 gene, which are known to be associated with cardiometabolic health.[39] We showed that these plasmalogens likely could not be validated in ARIC because their impact on ASCVD risk is dampened in older subjects.

The inverse association of *alpha-ketobutyrate* with ASCVD events, in both Heart SCORE and validation in ARIC study, represents a significant finding in humans. To our knowledge, such an association has not been observed in human population data before. Alpha-ketobutyrate is involved in the metabolism of several amino acids (e.g., homocysteine) and generated as a byproduct of cystathionine hydrolysis (into cysteine) via the transsulfuration pathway.[41,42] The conversion of alpha-ketobutyrate to its byproduct alpha-hydroxybutyrate indicates early signs of insulin resistance.[43] Using proteomics and network analysis to assess vein graft kinetics in mice, Decano et al., showed that tricarboxylic acid substrate utilisation of

Metabolite	OR in Heart SCORE [95% CI]	OR in ARIC [95% CI]	Adjusted p-value in Heart SCORE	Adjusted p-value in ARIC
1-(1-enyl-palmitoyl)-2-arachidonoyl-GPC (P-16:0/20:4)	0.54 [0.40, 0.74]	0.78 [0.47, 1.28]	0.040	1
1-(1-enyl-palmitoyl)-2-arachidonoyl-GPE (P-16:0/20:4)	0.57 [0.42, 0.78]	1.29 [0.87, 1.92]	0.077	1
1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)	0.58 [0.42, 0.80]	N/A	0.077	N/A
Pentadecanoate (15:0)	1.79 [1.31, 2.45]	N/A	0.069	N/A
1-stearoyl-2-oleoyl-GPE	0.60 [0.45, 0.81]	1.30 [1.01, 1.68]	0.077	0.39
1-palmitoyl-2-linoleoyl-GPI	**0.62 [0.47, 0.83]**	**0.75 [0.65, 0.87]**	**0.077**	**8.1×10^{-4}**
Alpha-ketobutyrate	**0.62 [0.49, 0.79]**	**0.86 [0.78, 0.94]**	**0.045**	**0.017**
2-oxoarginine	0.63 [0.48, 0.82]	1.07 [0.83, 1.38]	0.077	1
$C_{16}H_{18}N_2O_5$	0.50 [0.38, 0.65]	1.02 [0.84, 1.25]	5.3×10^{-4}	1
X-24295	0.63 [0.49, 0.82]	1.01 [0.90, 1.14]	0.075	1
X-14056	0.61 [0.46, 0.81]	0.88 [0.69, 1.13]	0.077	1
X-19438	0.66 [0.52, 0.84]	N/A	0.077	N/A

A Metabolite is bolded if it was successfully replicated in the ARIC study. An "N/A" means that metabolite was not available in the ARIC study.

Table 3: **Plasma metabolites and risk for incident atherosclerotic cardiovascular disease (ASCVD) events.**

α-ketobutyrate showed a trend of increasing rate on pemafibrate treatment and reversal on PPARα (peroxisome proliferator-activated receptors) silencing resulting in less vein graft lesions and failure.[44] Recently, Wu et al., reported that exogenous alpha-ketobutyrate supplementation promoted longevity and delay senescence in fibroblast cells via augmentation of NAD^+ and peroxisome biogenesis in *C. elegans* via the SIRT-NRF2 pathway.[45] It is, thus, plausible that alpha-ketobutyrate levels may form a favourable equilibrium between oxidative stress response and endothelial injury. Whether this is a direct effect of alpha-ketobutyrate by

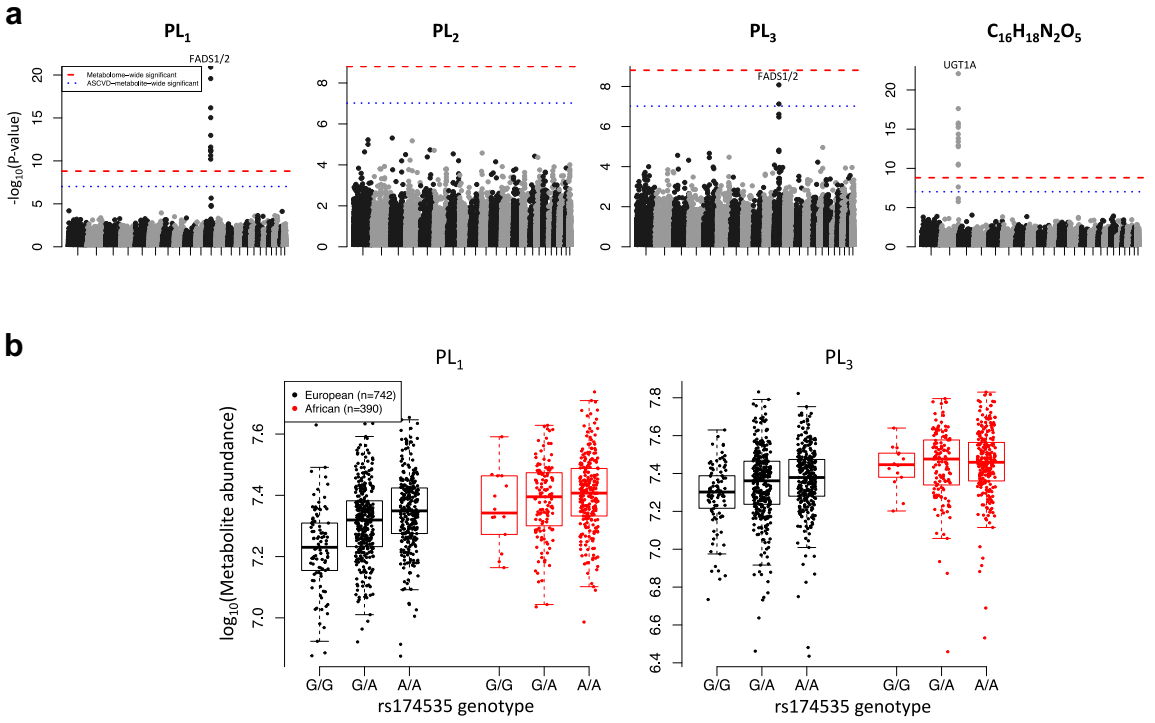

Fig. 3: Results of the Genome-Wide Association Study for Significant Metabolites. This figure presents the results of genome-wide association studies (GWAS) performed on the significant metabolites identified in the Heart SCORE study. Loci significantly associated with metabolite concentrations are annotated and mapped, providing insights into genetic contributions.

glutathione generation and perioxosomal proliferation or in part secondary to catabolism of homocysteine and reduction in its atherogenic effect, needs to be explored further.

Similar to alpha-ketobutyrate, the association of *1-palmitoyl-2-linoleoyl-GPI*, a specific metabolite of the PI lipid species, with ASCVD events has not been previously reported. PI lipids have a well-established role in cellular signalling, regulating humoral factors and vascular remodelling. PIs are predominantly involved in the inflammatory aspects of atherosclerosis[46]–inhibition of the PI3Kc-PIP3-Akt signalling pathway in murine models of atherosclerosis (ApoE- or LDLR-deficient mice) significantly reduces the development of early and advanced atherosclerotic lesions.[47–49] PIs are also involved in increased efflux of cholesterol from peripheral tissues to HDL and an increased transport and clearance of cholesterol through the liver and bile.[50]

Our findings of an inverse association of mid-life levels of bioactive *plasmalogens* with long-term ASCVD events in the Heart SCORE study are significant. Our findings extend those of Meike et al. showed that phosphatidylethanolamine (PE) plasmalogen species were inversely associated with unstable coronary artery disease but not with stable coronary disease in a cross-sectional study.[51] Plasmalogens are naturally occurring glycerophospholipids primarily present as phosphatidylcholine and phosphatidylethanolamine (PE) species.[52] PE plasmalogens are known to prevent oxidation of cholesterol in phospholipid bilayers[53] and are more abundantly present in the cardiovascular system. Plasmalogens' anti-oxidant potential[16] may be due to the enhanced electron density of the vinyl ether bond at the sn-1 position that make it susceptible to cleavage by reactive oxygen species.[52,54,55] The oxidative products of plasmalogens are unable to further propagate lipid peroxidation and may terminate the lipid oxidation process.[56] Our interaction analysis (by age $\geq$ 65 years) (Supplemental Table iii) showed a dampened effect of plasmalogens in older Heart SCORE participants. Given that the ARIC population were significantly older than the Heart SCORE study (mean age 75 y vs 59 y), it is plausible that the protective effect and generation of plasmalogens is more robust in younger individuals. We postulate that a potential cardioprotective role of plasmalogens in mid-life may be related to preventing lipid oxidation,[57] which modulates inflammation[7,58] and the initiation of atherosclerosis.[52,59,60]

Our plasmalogen findings are supported by the results of our metabolome-wide genetic association analysis. Two of the three plasmalogen metabolites inversely associated with future ASCVD events were strongly influenced by the genotype at *rs174535* located on the *FADS1/FADS2* genes. While, located in the gene body of MYRF, rs174535 is not an expression quantitative trait loci (eQTL) for MYRF in whole blood, liver, or heart, but is instead an eQTL for FADS1 and FADS2 in those tissues, where FADS1/2 lies 40 kilobases upstream from rs174535. Although we did not find statistically significant associations between the SNPs on or near FADS1/FADS2 and ASCVD events, we observed that estimates were in the expected direction, with more copies of *rs174535*'s G allele rendering a greater risk of ASCVD. Although this is a unique association, FADS1/FADS2-related SNPs have been linked with lipid level regulation.[61–63] The SNP *rs174535,* which is most associated with 1-(1-enyl-palmitoyl)-2-arachidonoyl-GPC and one of two SNPs associated with 1-(1-enyl-stearoyl)-2-arachidonoyl-GPE, is in near perfect linkage disequilibrium (r^2 = 0.98 in Europeans) with rs174547 and rs174546. These SNPs' minor alleles have been shown to increase the risk of aortic stenosis in European[38] and coronary artery disease and ischaemic stroke in Chinese populations,[39] respectively.

We also identified a strong inverse ASCVD association with $C_{16}H_{18}N_2O_5$, a bilirubin degradation product. This finding is consistent with prior reports of negative causal associations of total bilirubin levels with ASCVD.[64,65] These findings may be attributed to bilirubin's anti-oxidant properties as a superoxide scavenger, modulation of PPAR, and cytoprotective properties.[66,67]

Study limitations

Our study has some notable limitations. First, frozen plasma samples were used for analysis, which could have influenced measured concentrations of metabolites and lipids. However, we used contemporary quantitative *-omic* techniques unlikely to be affected by storage.[68] Second, metabolite concentrations are known to vary and we present one time-point metabolomic measurements. Although the robustness of our findings is strengthened by validation in an independent cohort, the effect of serially measured alpha-ketobutyrate, PI and plasmalogen metabolites on ASCVD risk may provide additional insight into our findings. Third, more precise contributions of environmental exposure that may influence metabolomic profiles were not included in this analysis. Fourth, although we show a modulation of alpha-ketobutyrate and PI levels by food intake, our dietary influences were self-reported and must be replicated in other datasets. Lipoprotein (a), which is increasingly recognised as a causal risk factor for ASCVD events, was not reliably tested in the Heart-SCORE population. Further, only a subset of significant metabolites from Heart SCORE study was available in the ARIC study visit 5 and thus plasmalogen metabolite 1-(1-enyl-palmitoyl)-2-arachidonoyl-GPE (P-16:0/20:4)] and pentadecanoate were not assessed. Finally, while both the Heart SCORE and ARIC studies include a robust representation of self-reported Black participants,[69] other minority groups are underrepresented. This limitation may affect the generalisability of our findings to those groups.

Conclusions

Our data indicate that higher mid-life levels of three plasmalogens, two amino acid metabolites, and a bilirubin degradation product, all of which have anti-inflammatory properties, are associated with lower risk of late-life ASCVD events, and modestly improve long-term risk prediction parameters. The biological plausibility of the protective role discovery of *alpha-ketobutyrate* and *1-palmitoyl-2-linoleoyl-GPI* are strengthened by the validation in a separate longitudinal cohort of biracial participants and may be regulated by dietary intake. Further research is needed to determine the causality and preventive therapeutic potential of these metabolites for ASCVD.

Contributors

Anum Saeed—study conceptualisation, interpretation, writing and editing drafted manuscript, corresponding author.

Chris McKennan—statistical methods and analysis, interpretation, writing and editing drafted manuscript.

Jiaxuan Duan—statistical analysis (Heart SCORE study).

Yueh-Ning Yang–statistical analysis (ARIC study).

Kevin E. Kip–reviewing and editing drafted manuscript.

David Finegold–reviewing and editing drafted manuscript.

Michael Vu—data interpretation, reviewing and editing drafted manuscript.

Justin Swanson–reviewing and editing drafted manuscript.

Oscar L. Lopez–reviewing and editing drafted manuscript.

Ann Cohen–reviewing and editing drafted manuscript.

Mark Mapstone–reviewing and editing drafted manuscript Bing Yu–validation study co-investigator, reviewing and editing drafted manuscript.

Christie M Ballantyne–validation study co-investigator, reviewing and editing drafted manuscript.

Steven E. Reis—funding for data collection, study conceptualisation, reviewing and editing drafted manuscript.

All authors have read and agree with the results of the final version of the manuscript.

Data sharing statement

Deidentified participant data underlying this article will be made available to researchers upon reasonable request, contingent on approval by the study's institutional review board and in alignment with ethical considerations. Requests should be directed to Anum Saeed, MD at saeeda@pitt.edu. Access will be granted for research purposes only, and a data use agreement may be required.

Declaration of interests

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105551.

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