# Metabolic Predictors of Incident Coronary Heart Disease in Women

**Nina P. Paynter, PhD**[1], **Raji Balasubramanian, ScD**[2], **Franco Giulianini, PhD**[1], **Dong D. Wang, MD DSc**[3,4], **Lesley F. Tinker, PhD**[5], **Shuba Gopal, PhD**[6], **Amy A. Deik, BA**[6], **Kevin Bullock, MS**[6], **Kerry A. Pierce, MLA**[6], **Justin Scott, BS**[6], **Miguel A. Martínez-González, MD PhD MPH**[4,7], **Ramon Estruch, MD PhD**[8], **JoAnn E. Manson, MD DrPH**[1,3], **Nancy R. Cook, ScD**[1,3], **Christine M. Albert, MD MPH**[1], **Clary B. Clish, PhD**[6], and **Kathryn M. Rexrode, MD MPH**[1,9]

[1]Division of Preventive Medicine, Brigham and Women's Hospital, Boston, MA

[2]Department of Biostatistics and Epidemiology, School of Public Health and Health Sciences, University of Massachusetts, Amherst, MA

[3]Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, MA

[4]Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, MA

[5]Fred Hutchinson Cancer Research Center, Seattle, WA

[6]Broad Institute of MIT and Harvard, Cambridge, MA

[7]Department of Preventive Medicine & Public Health, University of Navarra Medical School, Pamplona, Spain

[8]Department of Internal Medicine, Institut d'Investigacions Biomèdiques August Pi Sunyer, Hospital Clínic, University of Barcelona, Barcelona, Spain

[9]Division of Women's Health, Brigham and Women's Hospital, Boston, MA

## Abstract

**Background**—Although metabolomic profiling offers promise for prediction of coronary heart disease (CHD), and metabolic risk factors are more strongly associated with CHD in women than men, limited data are available for women.

**Methods**—We applied a liquid chromatography-tandem mass spectrometry metabolomics platform to measure 371 metabolites in a discovery set of postmenopausal women (472 incident CHD cases, 472 controls) with validation in an independent set of postmenopausal women (312 incident CHD cases, 315 controls).

**Results**—Eight metabolites, primarily oxidized lipids, were significantly dysregulated in cases after adjustment for matching and CHD risk factors in both the discovery and validation datasets.

Corresponding Author: Nina P. Paynter, npaynter@partners.org, Brigham and Women's Hospital, 900 Commonwealth Avenue East, 3rd Floor, Boston, MA 02215, Phone: 617-278-0798, Fax: 617-731-3843.

**Disclosures**
None

One oxidized phospholipid, C34:2 hydroxy-phosphatidylcholine (C34:2 hydroxy-PC), remained associated with CHD after further adjustment for other validated metabolites. Subjects with C34:2 hydroxy-PC levels in the highest quartile had a 4.7-fold increase in CHD odds compared to the lowest quartile; C34:2 hydroxy-PC also significantly improved the AUC (p<0.01) for CHD. The C34:2 hydroxy-PC findings replicated in a third replication dataset of 980 men and women (230 cardiovascular events) with a stronger association observed in women.

**Conclusions—**These data replicate known metabolite predictors, identify novel markers, and support the relationship between lipid oxidation and subsequent CHD.

## Keywords

metabolomics; women; coronary heart disease risk; oxidized lipids

## Introduction/Background

Coronary heart disease remains the leading cause of death in women, as in men. However, the clinical manifestations of CHD differs, with a greater preponderance of non-obstructive atherosclerosis and microvascular disease[1] and higher case fatality rates in women as compared to men. Metabolic risk factors, especially diabetes, are particularly strong risk factors for CHD in women. Women with type 2 diabetes have over 40% higher risk of CHD than men with diabetes[2, 3]. However, data on the relationship of metabolites and CHD using metabolomic profiling have been very limited in women.

Examinations of the relationship of CHD with circulating blood metabolite levels measured using metabolomics have illuminated different potential pathways to CHD development. Incident cardiovascular disease and longevity have been associated with metabolites in the citric acid cycle as well as gut microbe-associated trimethylamine N-oxide (TMAO), choline, and multiple lipid-related metabolites[4–11]. Glutamate and glutamine have also been associated with diabetes[12] as well as with cardiovascular disease in previous studies[4, 6, 9], as have branched chain and aromatic amino acids[12, 13]. However, despite evidence of sexual dimorphism in the metabolome[14, 15], these associations have not been examined in detail in women. Further, metabolite profiles may also change during menopause[16], a time coinciding with increased CHD risk. Therefore, the examination of metabolomics profiles in women may help to identify new metabolic associations and mechanistic pathways underlying CHD in women, which may extend also to men.

Here, we used metabolomics to identify novel metabolic signatures for incident CHD in female participants of the Women's Health Initiative[17], with independent discovery and validation sets derived from the Observational Study (WHI-OS)[18] and the placebo arms of the Hormone Trials (WHI-HT)[19], respectively. We also performed external replication using an independent mixed sex study from the Prevención con Dieta Mediterránea (PREDIMED) Trial[20, 21] to assess generalizability of significant metabolites to cardiovascular disease and both sexes.

## Methods

This study was approved by Partners Human Research Committee, which is the IRB of Brigham and Women's Hospital. Metabolite data from the discovery and validation datasets used in this study have been deposited in and are available from the dbGaP database under dbGaP accession phs001334.v1.p3.[22] All other data will not be made available by the authors, nor will analytic methods or study materials.

### Human Studies

**WHI-OS (Discovery)**—The discovery set was drawn from the Women's Health Initiative-Observational Study (WHI-OS). The full WHI-OS consisted of 93,676 postmenopausal women ineligible or unwilling to participate in the related hormone trials, enrolled between 1994 and 1998 in the US. All participants provided written informed consent. For this study, 472 participants who developed CHD after the baseline examination (cases) were selected, including all non-white cases with an eligible control and a sample of the white cases, and 472 participants who did not develop CHD frequency matched on 5-year age, race/ethnicity, hysterectomy status, and 2-year enrollment groups. Only 21 hormone therapy users were included in the discovery set and use was not adjusted for, but a sensitivity analysis removing the hormone therapy users did not change the effect estimates.

**WHI-HT (Validation)**—The validation set was drawn from the placebo arms of the Women Health Initiative Hormone Therapy Trials. One of the trials randomized 16,608 postmenopausal women to estrogen plus progesterone or placebo, while the other randomized 10,739 women with prior hysterectomy to estrogen or placebo. All participants provided written informed consent. We selected 312 cases and 315 frequency-matched controls from the two placebo arms with matching on 5-year age, race/ethnicity, hysterectomy status, and 2-year enrollment window.

**PREDIMED (Replication)**—The replication set was drawn from the Prevención con Dieta Mediterránea (PREDIMED) trial which also utilized the Broad Institute metabolomics platform. The full PREDIMED trial randomized men and women at high risk of developing cardiovascular disease to one of 3 interventions: a Mediterranean dietary intervention supplemented with virgin olive oil, a Mediterranean dietary intervention supplemented with nuts, or advice about a low-fat diet. All participants provided written informed consent. The primary endpoint for this trial was a composite of MI, stroke and CVD death. For this study, a case-cohort design as outlined by Prentice[23] was used, which included all 230 cases (74 MI, 115 stroke, and 31 CVD death) and a random 10% sample of participants at baseline (787 participants). Of the 787 sub-cohort participants, 37 had a primary endpoint during follow-up (and thus were also included in the 230 cases) while 750 did not, resulting in 980 unique participants for analysis.

### Metabolomics

Plasma samples were collected using EDTA tubes in the WHI and PREDIMED cohorts and processed immediately. All WHI specimens were stored in a −70° freezer within 2 hours of collection or stored at −20° for up to 2 days and shipped on dry ice and stored at −70° until

processing. The majority of the WHI samples had been thawed once prior to our study, with 7 samples (6 cases and 1 control) having been thawed twice. PREDIMED samples were shipped at −4°, and then stored at −70° until analysis. Metabolomic measurements were made using four complimentary LC-MS methods resulting in 371 metabolites for the WHI samples. Only the HILIC-positive and C8-positive methods were available in the PREDIMED samples. For each method, pooled plasma reference samples were included every 20 samples and results were standardized using the ratio of the value of the sample to the value of the nearest pooled reference multiplied by the median of all reference values for the metabolite.

HILIC analyses of water soluble metabolites in the positive ionization mode (HILIC-pos) were conducted using an LC-MS system comprised of a Shimadzu Nexera X2 U-HPLC (Shimadzu Corp.; Marlborough, MA) coupled to a Q Exactive hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific; Waltham, MA). Plasma samples (10 μL) were prepared via protein precipitation with the addition of nine volumes of 74.9:24.9:0.2 v/v/v acetonitrile/methanol/formic acid containing stable isotope-labeled internal standards (valine-d8, Sigma-Aldrich; St. Louis, MO; and phenylalanine-d8, Cambridge Isotope Laboratories; Andover, MA). The samples were centrifuged (10 min, $9,000 \times g$, 4°C), and the supernatants were injected directly onto a $150 \times 2$ mm, 3 μm Atlantis HILIC column (Waters; Milford, MA). The column was eluted isocratically at a flow rate of 250 μL/min with 5% mobile phase A (10 mM ammonium formate and 0.1% formic acid in water) for 0.5 minute followed by a linear gradient to 40% mobile phase B (acetonitrile with 0.1% formic acid) over 10 minutes. MS analyses were carried out using electrospray ionization in the positive ion mode using full scan analysis over 70–800 m/z at 70,000 resolution and 3 Hz data acquisition rate. Other MS settings were: sheath gas 40, sweep gas 2, spray voltage 3.5 kV, capillary temperature 350°C, S-lens RF 40, heater temperature 300°C, microscans 1, automatic gain control target 1e6, and maximum ion time 250 ms.

HILIC analyses of water soluble metabolites in the negative ionization mode (HILIC-neg) were conducted using an LC-MS system comprised of an AQUITY UPLC system (Waters; Milford, MA and a 5500 QTRAP mass spectrometer (SCIEX; Framingham, MA). Plasma samples (30 μL) were prepared via protein precipitation with the addition of four volumes of 80% methanol containing inosine-15N4, thymine-d4 and glycocholate-d4 internal standards (Cambridge Isotope Laboratories; Andover, MA). The samples were centrifuged (10 min, $9,000 \times g$, 4°C), and the supernatants were injected directly onto a $150 \times 2.0$ mm Luna NH2 column (Phenomenex; Torrance, CA). The column was eluted at a flow rate of 400 μL/min with initial conditions of 10% mobile phase A (20 mM ammonium acetate and 20 mM ammonium hydroxide in water) and 90% mobile phase B (10 mM ammonium hydroxide in 75:25 v/v acetonitrile/methanol) followed by a 10 min linear gradient to 100% mobile phase A. MS analyses were carried out using electrospray ionization and selective multiple reaction monitoring scans in the negative ion mode. To create the method, declustering potentials and collision energies were optimized for each metabolite by infusion of reference standards. The ion spray voltage was −4.5 kV and the source temperature was 500°C.

Positive ion mode analyses of polar and non-polar plasma lipids (C8-pos) were conducted using an LC-MS system comprised of a Shimadzu Nexera X2 U-HPLC (Shimadzu Corp.;

Marlborough, MA) coupled to a Exactive Plus orbitrap mass spectrometer (Thermo Fisher Scientific; Waltham, MA). Plasma samples (10 μL) were extracted for lipid analyses using 190 μL of isopropanol containing 1,2-didodecanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids; Alabaster, AL). After centrifugation, supernatants were injected directly onto a $100 \times 2.1$ mm, 1.7 μm ACQUITY BEH C8 column (Waters; Milford, MA). The column was eluted isocratically with 80% mobile phase A (95:5:0.1 vol/vol/vol 10mM ammonium acetate/methanol/formic acid) for 1 minute followed by a linear gradient to 80% mobile-phase B (99.9:0.1 vol/vol methanol/formic acid) over 2 minutes, a linear gradient to 100% mobile phase B over 7 minutes, then 3 minutes at 100% mobile-phase B. MS analyses were carried out using electrospray ionization in the positive ion mode using full scan analysis over 200–1000 m/z at 70,000 resolution and 3 Hz data acquisition rate. Other MS settings were: sheath gas 50, in source CID 5 eV, sweep gas 5, spray voltage 3 kV, capillary temperature 300°C, S-lens RF 60, heater temperature 300°C, microscans 1, automatic gain control target 1e6, and maximum ion time 100 ms. Lipid identities were determined based on comparison to reference plasma extracts and were denoted by total number of carbons in the lipid acyl chain(s) and total number of double bonds in the lipid acyl chain(s).

Negative ion mode analyses of free fatty acids and bile acids (C18-neg) were conducted using an LC-MS system comprised of a Shimadzu Nexera X2 U-HPLC (Shimadzu Corp.; Marlborough, MA) coupled to a Q Exactive hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific; Waltham, MA). Plasma samples (30 μL) were extracted using 90 uL of methanol containing PGE2-d4 (Cayman Chemical Co.; Ann Arbor, MI) and centrifuged (10 min, $9,000 \times g$, 4°C). The samples were injected onto a $150 \times 2$ mm ACQUITY T3 column (Waters; Milford, MA). The column was eluted isocratically at a flow rate of 400 μL/min with 60% mobile phase A (0.1% formic acid in water) for 4 minutes followed by a linear gradient to 100% mobile phase B (acetonitrile with 0.1% formic acid) over 8 minutes. MS analyses were carried out in the negative ion mode using electrospray ionization, full scan MS acquisition over 200–550 m/z, and a resolution setting of 70,000. Metabolite identities were confirmed using authentic reference standards. Other MS settings were: sheath gas 45, sweep gas 5, spray voltage –3.5 kV, capillary temperature 320°C, S-lens RF 60, heater temperature 300°C, microscans 1, automatic gain control target 1e6, and maximum ion time 250 ms.

Raw data from Q Exactive/Exactive Plus instruments were processed using TraceFinder software (Thermo Fisher Scientific; Waltham, MA) and Progenesis QI (Nonlinear Dynamics; Newcastle upon Tyne, UK) while MultiQuant (SCIEX; Framingham, MA) was used to process 5500 QTRAP data. For each method, metabolite identities were confirmed using authentic reference standards or reference samples. CVs were calculated using pooled plasma samples from the first 800 WHI-OS participants.

## Statistical Analysis

Two sets of covariates were used in WHI analyses. The first set included the frequency matching factors (age, race/ethnicity, hysterectomy status and enrollment window). The second set included the matching factors plus traditional CHD risk factors (smoking, systolic blood pressure, diabetes, total and HDL cholesterol) and CHD medication use (aspirin,

statins, anti-hyperglycemics and anti-hypertensives). All metabolites were scaled and log-normalized and missing values below the limit of detection were assigned to half the lowest observed value. R was used for all analyses with packages noted below.

**Discovery methods**—For the discovery analysis in the WHI-OS, each metabolite was examined individually in logistic models including both sets of covariates. Statistically significant associations satisfied FDR adjusted p<0.05 using the two-stage step-up procedure[24] – this procedure allows for dependencies between the metabolites and is implemented using the corresponding "TSBH" option of the *mt.rawp2adjp* function in the *multtest* package with a nominal alpha of 0.05. Unconditional logistic regression was chosen over conditional to improve precision of estimates while still controlling for the frequency matching factors[25].

**Validation methods**—Metabolite associations with CHD discovered in WHI-OS were validated in the independent WHI-HT data set. Metabolites were examined individually using the same covariate sets used for discovery – thus, a metabolite that was selected in the discovery process when adjusted for matching factors but not when adjusted for additional risk factors would only be validated with adjustment for matching factors. Metabolite associations were considered to have validated if the FDR-adjusted p value (same procedure as discovery) for the odds ratio after adjustment was less than 0.05 and the nominal p value was less than 0.025.

**AUC Calculation**—By combining data from the parent WHI cohort with that generated in the matched case control study, we estimated the prediction score for each subject using methods described by Qian and colleagues[26]. To estimate the Receiver Operating Characteristics (ROC) curve and associated AUC, we reweighted our sample to account for the frequency matched selection of cases and controls from the source populations in the WHI. We used 1000 bootstrap samples that preserved the frequency matching to construct the confidence interval for the difference in the AUC comparing models with and without C34:2 hydroxy-PC in addition to matching and other risk factors.

**Derived networks**—A correlation network was generated using the full WHI dataset in which nodes represent individual metabolites and edges between node pairs indicate a partial correlation (adjusting for case status) that exceeded 0.7 in magnitude. This procedure resulted in a network of 371 nodes and 1256 edges. The connected components of the partial correlation network were identified using the *clusters* function in the R *igraph* package. Fishers exact tests were then used to test whether each connected set of metabolites included more validated metabolites associated with CHD than expected by chance.

**Enantiomeric composition**—Since the reversed phase separation used in the LC-MS method used to profile the WHI dataset does not distinguish stereoisomers, enantiomeric purity was investigated using chiral chromatography following the method of Oh et al[27]. LC-MS tracings for representative samples from the WHI dataset were compared to enantiomerically pure standards as well as a racemic mixture. An Agilent 4695 mass spectrometer was used to measure the characteristic negative ion mode 319.2/115.1 transition at a collision energy setting of 13.

**Replication methods**—Four metabolites from the two available metabolomics platforms in PREDIMED were examined individually in weighted Cox proportional hazards models, adjusting for age, sex, intervention group, systolic blood pressure, total cholesterol, HDL cholesterol, diabetes, smoking, and statin use. The Barlow weighting method[28] was used to account for the random, non-stratified sampling design along with the exact case-cohort pseudolikelihood estimators with robust variance estimators, as proposed by Langholz and Jiao [29] for the Cox proportional hazards models. Metabolite associations were considered to have replicated if the p value for the hazard ratio was less than 0.05. Interaction p-values were determined using the Wald test for the interaction coefficient.

## Results

The baseline characteristics of the 944 women in the WHI-OS discovery set (472 cases and 472 controls) and the 627 women in the WHI-HT validation set (312 cases and 315 controls) are shown in Table 1. The mean age was similar in both sets. The median time to CHD event for the cases, defined as myocardial infarction or death due to CHD, was 5.8 years in the discovery set and 3.7 years in the validation set. A total of 371 metabolites of known identity were profiled using liquid chromatography tandem mass spectroscopy (LC-MS) with a median CV of 6.28%, as measured in replicate quality control pooled plasma samples included throughout the analyses.

The relationship between individual metabolites and CHD was assessed using logistic regression controlling for matching variables only or matching variables plus CHD risk factors. In the discovery set, 60 of the 371 metabolites were identified as significant (two stage FDR<0.05) controlling for matching variables alone and 36 (17 also identified in the matching analysis) were identified controlling for matching plus CHD risk factors. Results of the discovery analysis are shown in Supplemental Table 1.

All metabolites discovered when controlling for matching variables were then tested in a separate validation set of 627 women from the placebo arms of WHI-HT (312 cases and 315 controls), and 33 were significantly associated with CHD (FDR p<0.05, nominal p<0.025). Similarly, 8 of the 36 metabolites discovered with adjustment for matching plus CHD risk factors remained significant in the independent validation dataset (nominal p<0.0014). Table 2 presents the results only for validated metabolites; the results for all discovered metabolites can be found in Supplemental Table 2. Of the 8 metabolites that were significantly associated with CHD after adjustment for traditional CHD risk factors, 7 were associated with increased risk: C34:2 hydroxy-phosphatidylcholine (hydroxy-PC), C36:4 hydroxy-PC, 5-hydroxyeicosatetraenoic acid (HETE), 15-HETE, 11-HETE, glutamate, and cytidine monophosphate (CMP). A single metabolite, glutamine, was associated with decreased risk of CHD. Associations between the 8 metabolites and other biomarkers and CHD risk factors are shown in Figure 1. Of note, none of the oxidized lipid metabolites (C34:2 hydroxy-PC, C36:4 hydroxy-PC, 5-HETE, 15-HETE, and 11-HETE) were correlated with C-reactive protein, lipoprotein(a), or Lp-PLA2 activity or mass. Coefficients of variation and percent of samples with values below the limit of detection for validated metabolites are provided in Supplemental Table 3.

When the 8 validated metabolites were mutually adjusted for in models controlling for matching factors and CHD risk factors, only C34:2 hydroxy-PC remained significantly associated with CHD case status in the WHI. Women in the highest quartile of C34:2 hydroxy-PC had an OR of CHD of 4.69 (2.84, 7.90) compared to those in the lowest quartile. The relationship between C34:2 hydroxy-PC and CHD remained consistent across subgroups of baseline characteristics as shown in Figure 2. In order to evaluate the impact on prediction of CHD, we estimated the prediction score by integrating metabolomics data from the matched case control study to existing data on risk factors available for the full WHI cohort[26]. We estimated the area under the receiver operator characteristic curve (AUC) by incorporating inverse-probability weights to re-weight the distribution of risk factors in the controls to reflect the full WHI-OS and WHI-HT cohorts. When C34:2 hydroxy-PC was added to the model with traditional CHD risk factors and matching factors, the AUC improved significantly from 0.76 to 0.79 with a change of 0.03 (95% CI: 0.01, 0.05, p<0.01).

To identify connections between validated metabolites, a correlation network of the 371 metabolites was constructed using all metabolite pairs. Edges between metabolite pairs were drawn to indicate an absolute Spearman partial correlation coefficient exceeding 0.7, while adjusting for case status. An enrichment analysis was layered on the partial correlation network to discover clusters of connected (correlated) metabolites that are enriched for associations with CHD. Six clusters included validated metabolites, and of these, only 1 cluster, shown in Figure 3, contained significantly more validated metabolites than would be expected by chance (p=3.185e-07). Each node in this cluster was an oxidized lipid, including 2 hydroxy-PCs and 4 HETEs, 3 of which were validated after full adjustment for risk factors and one of which (12-HETE) was validated with adjustment for matching factors only.

To determine whether the mono-HETEs associated with CHD were generated by enzymatic activity (single isomers) or nonenzymatic oxidation of arachidonic acid (racemic mixture), we analyzed stereoisomers of 5-HETE using chiral chromatography. As shown in Figure 4, enantiomerically pure 5(R)-HETE and 5(S)-HETE standards were measured at nominal retention times of 3.0 and 5.0 minutes, respectively, while a racemic mixture of 5(+/−)-HETE showed nearly equivalent measured peak areas between the enantiomers. Analyses of five representative plasma samples selected from the highest quartiles of 5-HETE levels in WHI showed generally similar 5(S) and 5(R) peak areas, suggesting that the WHI plasma measured levels of 5-HETE are a mixture of R- and S-enantiomers, in similar proportions.

Finally, we sought to test the generalizability of our findings in a new independent cohort, PREDIMED, that included both men and women and used a combined CVD endpoint of incident MI, stroke, and CHD death (230 cases) in a case-cohort design. Two of the four LC-MS methods done in the same lab used to profile the WHI data had been applied to PREDIMED, allowing us to test 4 of the 8 validated metabolites (glutamine, glutamate, C34:2 hydroxy-PC, and C36:4 hydroxy-PC). Baseline characteristics of the PREDIMED validation cohort are shown in Supplementary Table 4. For the combined CVD outcome in men and women, 3 of the 4 metabolites (both hydroxy-PCs and glutamate) replicated while glutamine did not (Table 3). All of the relationships were stronger for women, with only glutamate remaining significant in men alone. An interaction term indicated significant

effect modification by sex for both hydroxy-PCs (p for interaction <0.001). In a sensitivity analysis using CHD cases only (74 cases), the hazard ratio (HR) for C34:2 hydroxy-PC was 1.56 (1.09–2.24), P=0.02. For C36:4 hydroxy-PC the CHD only HR was 1.59 (1.10–2.30) P=0.01. In a corresponding analysis using stroke cases only (115 cases), the HR for C34:2 hydroxy-PC was 1.48 (1.13–1.92), P=0.004, and for C36:4 hydroxy-PC was 1.34 (1.02–1.77) P=0.036.

## Discussion

In our study of the association of metabolites with CHD in post-menopausal women, we identified and validated 8 metabolites associated with future CHD in women. These metabolites included glutamine, glutamate, cytidine monophosphate (CMP), and five oxidized lipids that had not previously been associated with incident CHD in humans. The strongest associations with CHD were observed for the oxidized lipids including two species of hydroxy-PCs (C34:2 and C36:4) and three oxidized derivatives from arachidonic acid (15-HETE, 5-HETE, and 11-HETE). The hydroxy-PCs were also externally validated for CVD, CHD and stroke in an external population (PREDIMED), with stronger associations seen in women.

Arachidonic acid derived HETEs have previously been associated with atherosclerosis, but not previously with incident CHD. A previous study by Oni-Orisan et al found an association between higher levels of 11-HETE and 15-HETE and extent of prevalent coronary artery disease.[30] 5-HETE, 12-HETE, and 15-HETE are well characterized products of 5-, 12-, and 15-lipoxygenase activity, respectively. 5-Lipoxgenase (ALOX5) activity is essential for leukotriene biosynthesis and is therefore linked to a number of inflammatory pathologies. However, when mono-HETEs are produced by these enzymes, the stereochemistry of the hydroxyl bound carbon is in the S-configuration. Our analyses found that the HETEs associated with CHD appeared to be produced from oxidative stress. This is the first report of an association between non-enzymatically generated mono-HETEs and incidence of CHD.

We also identified two novel oxidized phospholipids, C34:2 hydroxy-PC and C36:4 hydroxy-PC, associated with CHD. C34:2 hydroxy-PC was the metabolite most strongly associated with CHD in our cohort, with a more than 4-fold increased risk of CHD for women in the highest quartile. This association was robust across all strata tested, including whether or not aspirin and statins were used, or whether there was baseline diabetes or obesity. In PREDIMED, where the majority of incident CVD cases were in men, C34:2 hydroxy-PC was validated as significantly associated with CVD, as well as CHD and stroke. When examined by sex, women had a significant 79% increased risk of CVD per SD of C34:2 hydroxy-PC but results were nonsignificant in men, despite fewer CVD events occurring in women, and the sex by metabolite interaction term was significant. Similar associations were observed for C36:4 hydroxy-PC. Of note, no antioxidants were added to the samples to prevent ex-vivo oxidation in either of the studies. Whether these biomarkers remain more strongly associated with CVD in women in other populations, as well as the underlying mechanisms, requires further exploration.

The dysregulated oxidized lipids (HETEs and hydroxy-PCs) associated with CHD may offer new markers of oxidation and risk. They were not correlated with other biomarkers measured ($\rho < 0.2$) including inflammation markers, traditional lipids, BMI and systolic blood pressure, but are strongly associated with each other, as illustrated in the network analysis, and weakly associated with the other metabolites. From the stereoisomer analysis, the mono-HETE profiles are consistent with a non-enzymatic oxidative mechanism, which could also include generation of the hydroxy-PCs. While we were unable to assess replication of the HETEs in the PREDIMED due to the lack of available data on this class of metabolites, the strong replication of the hydroxy-PCs along with the strong correlation between the hydroxy-PCs and the HETEs suggests that the HETEs are likely to replicate as well. Lipid oxidation has been extensively studied in the context of model systems of overt atherosclerotic lesions[31]. Demonstration of the relationship between significantly dysregulated oxidized lipids and cardiovascular outcomes in longitudinal human cohort studies has been more difficult but some associations with CHD outcomes[32, 33] and mortality[34] have been shown. Additionally, a recent study of plasma metabolites of patients with existing CHD compared to healthy controls also identified lipid peroxidation related metabolites, including 5-HETE[35]. Our data support the hypothesis that early mechanisms involving lipid oxidation, for which these metabolites may be part of the cause or a consequence, may contribute to the etiology of CHD years before an event.

Our results are also not inconsistent with prior studies focusing narrowly on lipidomics. Both TAGs and DAGs were associated with CHD in our sample, though the association was eliminated after adjustment for total and HDL cholesterol. Using a lipidomics platform, Stegemann et al, found that C54.2 TAG was associated with increased risk of CHD in the Bruneck Study, with validation in Twins UK.[7] Methods between their study and ours differed in the metabolites measured; additionally, ours included an increased number of cases for discovery and exclusively included women for the discovery and validation sets. We did replicate the C54:2 TAG finding, though not after adjustment for traditional risk factors, but did not find a significant association for C16:1CE, C36.5 PE, or C18:1 CE. Alshehry et al also found multiple lipids associated with future cardiovascular disease in a population with type 2 diabetes. Of these, we found similar nominal associations for C16:0 Ceramide(d18:1), C22:0 Ceramide(d18:1), C40:6 PC, and C56:6 TAG[36].

Direct associations for glutamate and inverse associations for glutamine, initially identified by similar metabolomic profiling, have been consistently reported for both CHD and as well as diabetes[4, 6, 9, 37]. Cheng et al found that glutamate and glutamine were associated with markers of glucose metabolism as well as incident diabetes and showed that glutamine supplementation improved glucose tolerance in mice[37]. A glutamic acid related polymorphism has also been related to a higher risk of incident CHD among diabetics[38]. Other metabolomic studies have also found associations of glutamate and glutamine with CHD including two studies primarily in men[6, 9] as well as one which replicated the association in samples with both men and women[4].

Of note, TMAO, choline and branched chain and aromatic amino acids previously identified as associated with increased CHD risk were not selected using the data-driven approach of our study. TMAO and choline were not nominally significant in this cohort of women

(Supplemental Table 1). Other studies have reported higher branch chain amino acid levels in men than women[14] and in a pre-specified PREDIMED analysis combining men and women, branched chain amino acid levels were associated with CVD events[39]. We did identify a relationship between risk of CHD and isocitrate in matched analyses, but other branched chain amino acids were not associated with CHD in our cohort. Isocitrate was inversely associated with longevity and positively associated with cardiovascular disease as reported by Cheng et al, in an analysis also using the Framingham dataset[11].

Our study has several strengths including a well-validated metabolomics platform, detailed covariate information, a large number of carefully adjudicated endpoints, and a robust methodology. In addition, the study provides a detailed look at metabolomics and CHD in women, which has previously been lacking. Our results did replicate in the similarly aged women of the PREDIMED study, where the mean BMI was similar. While the generalizability beyond these postmenopausal women remains to be seen, these women are representative of the current population BMI and, importantly, represent a group of women at high risk of CHD. Additional data will also be needed to identify whether these results are specific to women or menopausal status or whether there is a smaller effect in men, as seen in the PREDIMED data.

## Conclusion

In two separate sets of post-menopausal women, multiple metabolites associated with CHD risk were identified and validated. These metabolites included novel metabolites, such as hydroxy-PCs and HETEs, as well as glutamate and glutamine, which have been consistently identified in other populations. The strongest marker, C34:2 hydroxy-PC, further replicated in a third set of women and men, with a suggestion of an interaction by sex. Overall, the strong association of oxidized phospholipids (hydroxy-PCs and HETEs) with CHD supports additional investigation of the mechanism and potential clinical use of oxidative risk. Further evaluation of these markers in other populations, as well as more detailed biologic inquiry, is warranted.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

## References

1. Shaw LJ, Bairey Merz CN, Pepine CJ, Reis SE, Bittner V, Kelsey SF, Olson M, Johnson BD, Mankad S, Sharaf BL, Rogers WJ, Wessel TR, Arant CB, Pohost GM, Lerman A, Quyyumi AA, Sopko G. Insights From the NHLBI-Sponsored Women's Ischemia Syndrome Evaluation (WISE) Study: Part I: Gender Differences in Traditional and Novel Risk Factors, Symptom Evaluation, and Gender-Optimized Diagnostic Strategies. Journal of the American College of Cardiology. 2006; 47:S4–S20. http://dx.doi.org/10.1016/j.jacc.2005.01.072. [PubMed: 16458170]

2. Regensteiner JG, Golden S, Huebschmann AG, Barrett-Connor E, Chang AY, Chyun D, Fox CS, Kim C, Mehta N, Reckelhoff JF. Sex Differences in the Cardiovascular Consequences of Diabetes Mellitus. Circulation. 2015; 132:2424–2447. [PubMed: 26644329]

3. Peters SA, Huxley RR, Woodward M. Diabetes as a risk factor for stroke in women compared with men: a systematic review and meta-analysis of 64 cohorts, including 775 385 individuals and 12 539 strokes. The Lancet. 2014; 383:1973–1980.

4. Würtz P, Havulinna AS, Soininen P, Tynkkynen T, Prieto-Merino D, Tillin T, Ghorbani A, Artati A, Wang Q, Tiainen M. Metabolite profiling and cardiovascular event risk: a prospective study of three population-based cohorts. Circulation. 2015; 131:774–785. [PubMed: 25573147]

5. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, DuGar B, Feldstein AE, Britt EB, Fu X, Chung Y-M. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011; 472:57–63. [PubMed: 21475195]

6. Vaarhorst AA, Verhoeven A, Weller CM, Böhringer S, Göraler S, Meissner A, Deelder AM, Henneman P, Gorgels AP, van den Brandt PA. A metabolomic profile is associated with the risk of incident coronary heart disease. American heart journal. 2014; 168:45–52. e7. [PubMed: 24952859]

7. Stegemann C, Drozdov I, Shalhoub J, Humphries J, Ladroue C, Didangelos A, Baumert M, Allen M, Davies AH, Monaco C. Comparative lipidomics profiling of human atherosclerotic plaques. Circulation: Cardiovascular Genetics. 2011; 4:232–242. [PubMed: 21511877]

8. Shah SH, Sun J-L, Stevens RD, Bain JR, Muehlbauer MJ, Pieper KS, Haynes C, Hauser ER, Kraus WE, Granger CB. Baseline metabolomic profiles predict cardiovascular events in patients at risk for coronary artery disease. American heart journal. 2012; 163:844–850. e1. [PubMed: 22607863]

9. Shah, SH., Bain, JR., Muehlbauer, MJ., Stevens, RD., Crosslin, DR., Haynes, C., Dungan, J., Newby, LK., Hauser, ER., Ginsburg, GS. Association of a peripheral blood metabolic profile with coronary artery disease and risk of subsequent cardiovascular events. Circulation: Cardiovascular Genetics. 2010. https://doi.org/10.1161/CIRCGENETICS.109.852814

10. Ganna A, Salihovic S, Sundström J, Broeckling CD, Hedman ÅK, Magnusson PK, Pedersen NL, Larsson A, Siegbahn A, Zilmer M. Large-scale metabolomic profiling identifies novel biomarkers for incident coronary heart disease. PLoS Genet. 2014; 10:e1004801. [PubMed: 25502724]

11. Cheng S, Larson MG, McCabe EL, Murabito JM, Rhee EP, Ho JE, Jacques PF, Ghorbani A, Magnusson M, Souza AL, Deik AA, Pierce KA, Bullock K, O'Donnell CJ, Melander O, Clish CB, Vasan RS, Gerszten RE, Wang TJ. Distinct metabolomic signatures are associated with longevity in humans. Nature communications. 2015; 6:6791.

12. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, Lewis GD, Fox CS, Jacques PF, Fernandez C, O'Donnell CJ, Carr SA, Mootha VK, Florez JC, Souza A, Melander O, Clish CB, Gerszten RE. Metabolite profiles and the risk of developing diabetes. Nature medicine. 2011; 17:448–453.

13. Magnusson M, Lewis GD, Ericson U, Orho-Melander M, Hedblad B, Engström G, Östling G, Clish C, Wang TJ, Gerszten RE. A diabetes-predictive amino acid score and future cardiovascular disease. European heart journal. 2013; 34:1982–1989. [PubMed: 23242195]

14. Krumsiek J, Mittelstrass K, Do KT, Stückler F, Ried J, Adamski J, Peters A, Illig T, Kronenberg F, Friedrich N, Nauck M, Pietzner M, Mook-Kanamori DO, Suhre K, Gieger C, Grallert H, Theis FJ, Kastenmüller G. Gender-specific pathway differences in the human serum metabolome. Metabolomics. 2015; 11:1815–1833. [PubMed: 26491425]

15. Mittelstrass K, Ried JS, Yu Z, Krumsiek J, Gieger C, Prehn C, Roemisch-Margl W, Polonikov A, Peters A, Theis FJ. Discovery of sexual dimorphisms in metabolic and genetic biomarkers. PLoS Genet. 2011; 7:e1002215. [PubMed: 21852955]

16. Auro K, Joensuu A, Fischer K, Kettunen J, Salo P, Mattsson H, Niironen M, Kaprio J, Eriksson JG, Lehtimäki T. A metabolic view on menopause and ageing. Nature communications. 2014; 5:4708.

17. The Women's Health Initiative Study Writing Group. Design of the women's health initiative clinical trial and observational study. Controlled clinical trials. 1998; 19:61–109. [PubMed: 9492970]

18. Pradhan AD, Manson JE, Rossouw JE, Siscovick DS, Mouton CP, Rifai N, Wallace RB, Jackson RD, Pettinger MB, Ridker PM. Inflammatory biomarkers, hormone replacement therapy, and incident coronary heart disease: prospective analysis from the Women's Health Initiative observational study. Jama. 2002; 288:980–987. [PubMed: 12190368]

19. Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. Jama. 2002; 288:321–333. [PubMed: 12117397]

20. Estruch R, Ros E, Martinez-Gonzalez M. Mediterranean diet for primary prevention of cardiovascular disease. N Engl J Med. 2013; 369:676–677. [PubMed: 23944307]

21. Martínez-González MÁ, Corella D, Salas-Salvadó J, Ros E, Covas MI, Fiol M, Wärnberg J, Arós F, Ruíz-Gutiérrez V, Lamuela-Raventós RM. Cohort profile: design and methods of the PREDIMED study. International journal of epidemiology. 2012; 41:377–385. [PubMed: 21172932]

22. Rexrode, K. DbGap. National Library of Medicine; Metabolomics of Coronary Heart Disease (CHD) in the WHI. https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs001334.v1.p3

23. Prentice RL. A case-cohort design for epidemiologic cohort studies and disease prevention trials. Biometrika. 1986; 73:1–11.

24. Benjamini Y, Krieger AM, Yekutieli D. Adaptive linear step-up procedures that control the false discovery rate. Biometrika. 2006; 93:491–507.

25. Pearce N. Analysis of matched case-control studies. BMJ. 2016; 352:i969. [PubMed: 26916049]

26. Qian J, Payabvash S, Kemmling A, Lev MH, Schwamm LH, Betensky RA. Variable selection and prediction using a nested, matched case-control study: Application to hospital acquired pneumonia in stroke patients. Biometrics. 2014; 70:153–163. [PubMed: 24320930]

27. Oh SF, Vickery TW, Serhan CN. Chiral lipidomics of E-series resolvins: aspirin and the biosynthesis of novel mediators. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2011; 1811:737–747. [PubMed: 21712098]

28. Barlow WE, Ichikawa L, Rosner D, Izumi S. Analysis of case-cohort designs. Journal of clinical epidemiology. 1999; 52:1165–1172. [PubMed: 10580779]

29. Langholz B, Jiao J. Computational methods for case-cohort studies. Computational Statistics & Data Analysis. 2007; 51:3737–3748.

30. Oni-Orisan A, Edin ML, Lee JA, Wells MA, Christensen ES, Vendrov KC, Lih FB, Tomer KB, Bai X, Taylor JM. Cytochrome P450-derived epoxyeicosatrienoic acids and coronary artery disease in humans: a targeted metabolomics study. Journal of lipid research. 2016; 57:109–119. [PubMed: 26555503]

31. Lee S, Birukov KG, Romanoski CE, Springstead JR, Lusis AJ, Berliner JA. Role of phospholipid oxidation products in atherosclerosis. Circulation research. 2012; 111:778–799. [PubMed: 22935534]

32. Gómez M, Vila J, Elosua R, Molina L, Bruguera J, Sala J, Masià R, Covas MI, Marrugat J, Fitó M. Relationship of lipid oxidation with subclinical atherosclerosis and 10-year coronary events in general population. Atherosclerosis. 2014; 232:134–140. [PubMed: 24401227]

33. Koenig W, Karakas M, Zierer A, Herder C, Baumert J, Meisinger C, Thorand B. Oxidized LDL and the risk of coronary heart disease: results from the MONICA/KORA Augsburg Study. Clinical Chemistry. 2011; 57:1196–1200. [PubMed: 21697499]

34. Linna M, Ahotupa M, Löppönen MK, Irjala K, Vasankari T. Circulating oxidised LDL lipids, when proportioned to HDL-c, emerged as a risk factor of all-cause mortality in a population-based survival study. Age and ageing. 2013; 42:110–113. [PubMed: 22693160]

35. Lu J, Chen B, Chen T, Guo S, Xue X, Chen Q, Zhao M, Xia L, Zhu Z, Zheng L, Yin H. Comprehensive metabolomics identified lipid peroxidation as a prominent feature in human

plasma of patients with coronary heart diseases. Redox Biology. 2017; 12:899–907. [PubMed: 28472752]

36. Alshehry, ZH., Mundra, PA., Barlow, CK., Mellett, NA., Wong, G., McConville, MJ., Simes, J., Tonkin, AM., Sullivan, DR., Barnes, EH. Plasma lipidomic profiles improve upon traditional risk factors for the prediction of cardiovascular events in type 2 diabetes. Circulation. 2016. https://doi.org/10.1161/CIRCULATIONAHA.116.023233

37. Cheng S, Rhee EP, Larson MG, Lewis GD, McCabe EL, Shen D, Palma MJ, Roberts LD, Dejam A, Souza AL, Deik AA, Magnusson M, Fox CS, O'Donnell CJ, Vasan RS, Melander O, Clish CB, Gerszten RE, Wang TJ. Metabolite profiling identifies pathways associated with metabolic risk in humans. Circulation. 2012; 125:2222–2231. [PubMed: 22496159]

38. Qi L, Qi Q, Prudente S, Mendonca C, Andreozzi F, di Pietro N, Sturma M, Novelli V, Mannino GC, Formoso G. Association between a genetic variant related to glutamic acid metabolism and coronary heart disease in individuals with type 2 diabetes. JAMA. 2013; 310:821–828. [PubMed: 23982368]

39. Ruiz-Canela M, Toledo E, Clish CB, Hruby A, Liang L, Salas-Salvadó J, Razquin C, Corella D, Estruch R, Ros E. Plasma branched-chain amino acids and incident cardiovascular disease in the PREDIMED trial. Clinical chemistry. 2016; 62:582–592. [PubMed: 26888892]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

## Clinical Perspective

**What is new?**

- Using a robust design, we identified and validated 33 metabolites associated with coronary heart disease (CHD) in postmenopausal women, 8 of which remained independently associated after adjustment for standard cardiovascular risk factors.

- The risk association for the novel metabolite with the strongest association (C34:2 hydroxy-phosphatidylcholine) is independent of other metabolites, improves the AUC when added to standard risk factors, and replicates in an additional dataset containing both men and women as well as for both CHD and stroke events.

- We have identified a cluster of novel metabolites related to oxidative stress, which are not strongly correlated with traditional biomarkers.

**What are the clinical implications?**

- Identified metabolites may be useful as additional biomarkers for clinical risk assessment in addition to highlighting new areas of biologic exploration and potential therapeutic targets.

- Metabolite precursors of CHD need to be studied in sufficient samples sizes of both men and women.
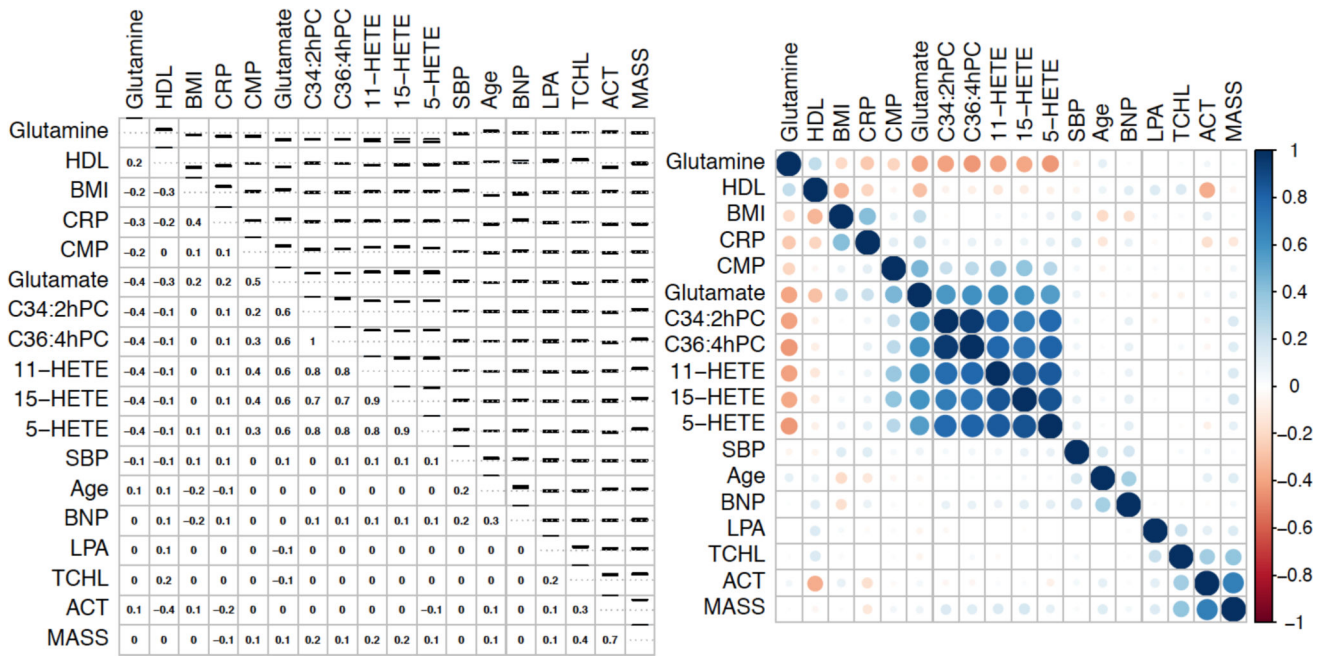
**Figure 1.**
Correlations between validated metabolites, cardiovascular biomarkers, age, BMI, and
systolic blood pressure. Left panel shows spearman correlations on bottom and confidence
interval on top; right panel shows heat map. Both figures are ordered using hierarchical
clustering. SBP: Systolic blood pressure, TCHL: Total cholesterol, HDL: HDL cholesterol,
BMI: Body mass index, CRP: log C-reactive protein, ACT: Lp-PLA2 activity, LPA:
Lipoprotein(a), MASS: Lp-PLA2 mass, BNP: N-terminal pro-BNP

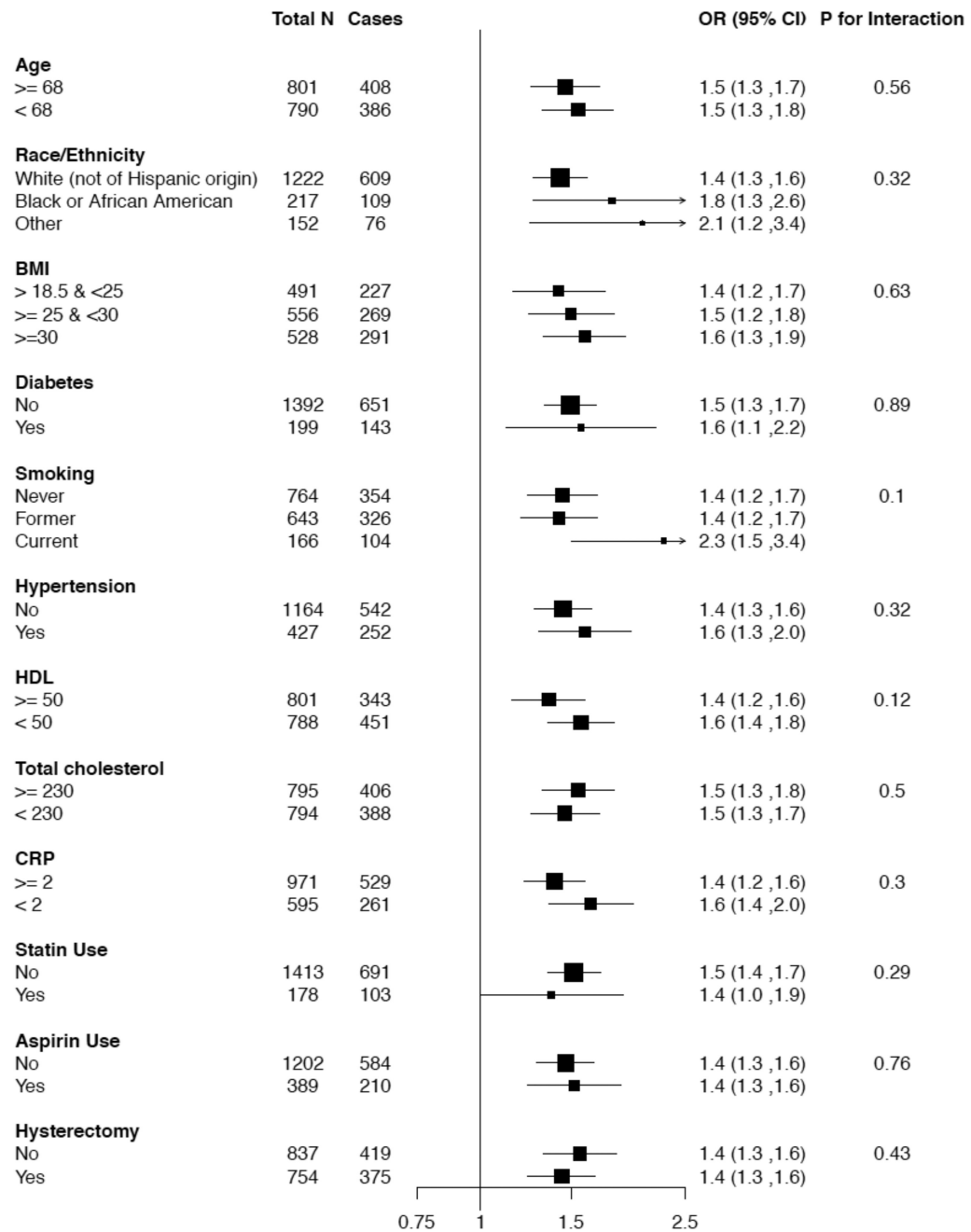| | Total N | Cases | | OR (95% CI) | P for Interaction |
|---|---|---|---|---|---|
| **Age** | | | | | |
| >= 68 | 801 | 408 | | 1.5 (1.3 ,1.7) | 0.56 |
| < 68 | 790 | 386 | | 1.5 (1.3 ,1.8) | |
| **Race/Ethnicity** | | | | | |
| White (not of Hispanic origin) | 1222 | 609 | | 1.4 (1.3 ,1.6) | 0.32 |
| Black or African American | 217 | 109 | | 1.8 (1.3 ,2.6) | |
| Other | 152 | 76 | | 2.1 (1.2 ,3.4) | |
| **BMI** | | | | | |
| > 18.5 & <25 | 491 | 227 | | 1.4 (1.2 ,1.7) | 0.63 |
| >= 25 & <30 | 556 | 269 | | 1.5 (1.2 ,1.8) | |
| >=30 | 528 | 291 | | 1.6 (1.3 ,1.9) | |
| **Diabetes** | | | | | |
| No | 1392 | 651 | | 1.5 (1.3 ,1.7) | 0.89 |
| Yes | 199 | 143 | | 1.6 (1.1 ,2.2) | |
| **Smoking** | | | | | |
| Never | 764 | 354 | | 1.4 (1.2 ,1.7) | 0.1 |
| Former | 643 | 326 | | 1.4 (1.2 ,1.7) | |
| Current | 166 | 104 | | 2.3 (1.5 ,3.4) | |
| **Hypertension** | | | | | |
| No | 1164 | 542 | | 1.4 (1.3 ,1.6) | 0.32 |
| Yes | 427 | 252 | | 1.6 (1.3 ,2.0) | |
| **HDL** | | | | | |
| >= 50 | 801 | 343 | | 1.4 (1.2 ,1.6) | 0.12 |
| < 50 | 788 | 451 | | 1.6 (1.4 ,1.8) | |
| **Total cholesterol** | | | | | |
| >= 230 | 795 | 406 | | 1.5 (1.3 ,1.8) | 0.5 |
| < 230 | 794 | 388 | | 1.5 (1.3 ,1.7) | |
| **CRP** | | | | | |
| >= 2 | 971 | 529 | | 1.4 (1.2 ,1.6) | 0.3 |
| < 2 | 595 | 261 | | 1.6 (1.4 ,2.0) | |
| **Statin Use** | | | | | |
| No | 1413 | 691 | | 1.5 (1.4 ,1.7) | 0.29 |
| Yes | 178 | 103 | | 1.4 (1.0 ,1.9) | |
| **Aspirin Use** | | | | | |
| No | 1202 | 584 | | 1.4 (1.3 ,1.6) | 0.76 |
| Yes | 389 | 210 | | 1.4 (1.3 ,1.6) | |
| **Hysterectomy** | | | | | |
| No | 837 | 419 | | 1.4 (1.3 ,1.6) | 0.43 |
| Yes | 754 | 375 | | 1.4 (1.3 ,1.6) | |

0.75    1    1.5    2.5

**Figure 2.**
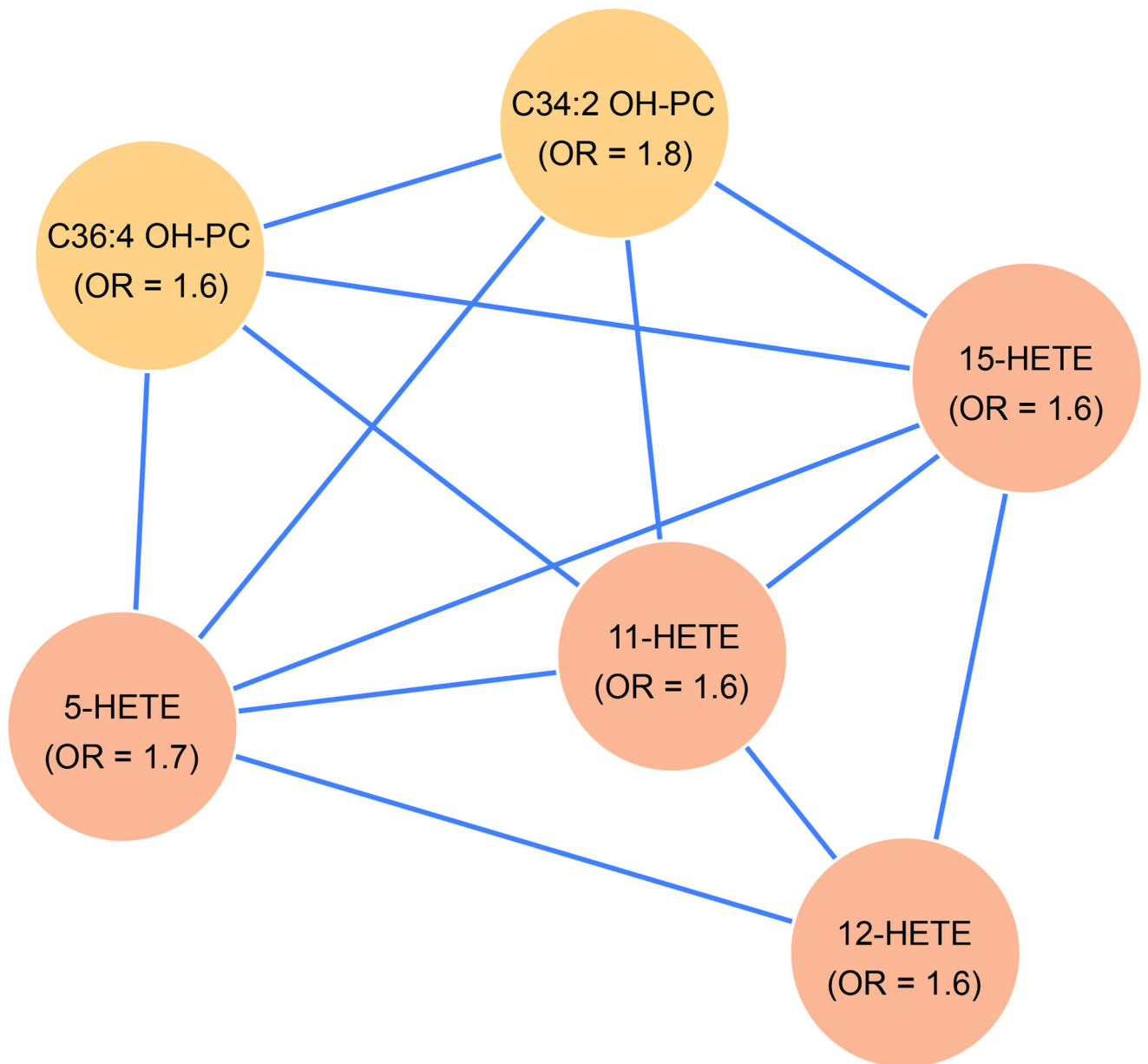Subgroup analysis for C34:2 hydroxy-PC in WHI-HT women

**Figure 3.**
Cluster of 6 oxidized lipid metabolites, all 6 of which are validated markers in a network of spearman partial correlations. Enrichment p value = 3.185e-07. All markers are positively associated with increased CHD risk. All correlations (edges) are positive.
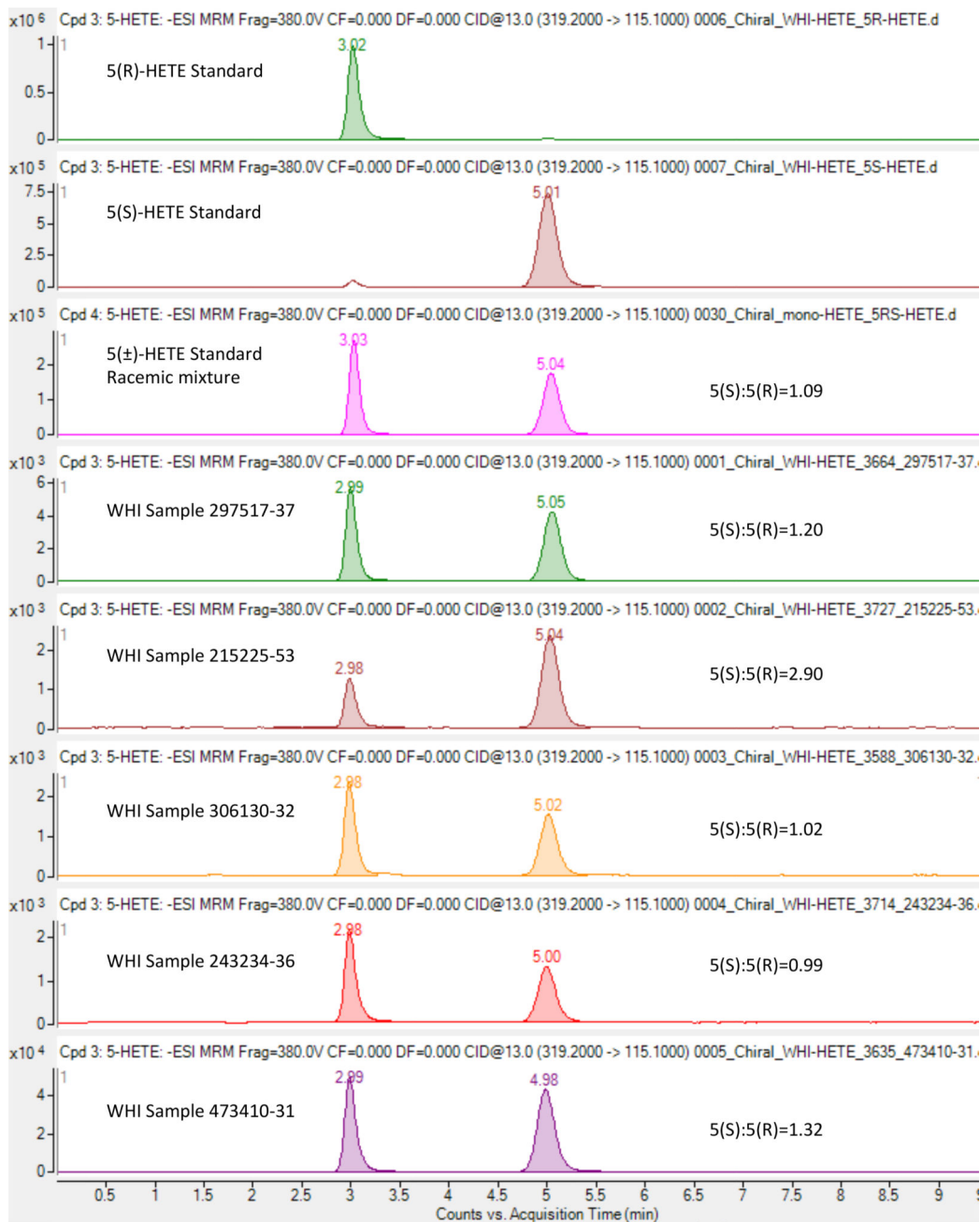
**Figure 4.**
Chiral chromatography analysis of 5-HETE. Panels (from top) are the results from agilent 4695 mass spectrometer measurements of the characteristic negative ion mode 319.2/115.1 transition at a collision energy setting of 13 for: (1) enantiomerically pure 5(R)-HETE; (2) enantiomerically pure 5(S)-HETE; (3) a racemic mixture of 5(+/−)-HETE; (4–8) five representative plasma samples selected from the highest quartiles of 5-HETE levels in WHI.

Baseline Characteristics of WHI Datasets

**Table 1**

| | WHI-OS Discovery (N=944) | | | WHI-HT Validation (N=627) | | |
|---|---|---|---|---|---|---|
| | Cases N = 472 | Controls N = 472 | p-value | Cases N = 312 | Controls N = 315 | p-value |
| **Age**, years | 68 (7) | 67 (7) | 0.51 | 67 (7) | 66 (7) | 0.64 |
| **Race** | | | 1.00 | | | 1.00 |
| White | 348 (73%) | 348 (73%) | | 254 (81%) | 256 (81%) | |
| Black | 69 (15%) | 69 (15%) | | 37 (12%) | 38 (12%) | |
| Other | 55 (12%) | 55 (12%) | | 21 (7%) | 21 (7%) | |
| **Systolic blood pressure**, mmHg | 135 (19) | 130 (18) | <0.001 | 137 (19) | 131 (18) | <0.001 |
| **Diabetes** | 78 (17%) | 25 (5%) | <0.001 | 65 (21%) | 31 (10%) | <0.001 |
| **BMI**, $m^2/kg$ | 29 (6) | 27 (6) | 0.001 | 30 (6) | 29 (6) | 0.21 |
| **Total cholesterol**, mg/dL | 231 (47) | 232 (47) | 0.51 | 236 (43) | 235 (39) | 0.57 |
| **HDL cholesterol**, mg/dL | 51 (16) | 57 (17) | <0.001 | 47 (12) | 52 (12) | <0.001 |
| **Smoking status,** | | | 0.016 | | | 0.033 |
| Current | 48 (10%) | 28 (6%) | | 56 (18%) | 34 (11%) | |
| Former | 209 (44%) | 243 (51%) | | 145 (46%) | 166 (53%) | |
| Never | 215 (46%) | 201 (43%) | | 111 (36%) | 115 (36%) | |
| **Aspirin use** | 116 (25%) | 98 (21%) | 0.16 | 93 (30%) | 78 (25%) | 0.16 |
| **Statin use** | 42 (9%) | 39 (8%) | 0.72 | 60 (19%) | 36 (11%) | 0.007 |
| **Anti-hypertensive use** | 144 (31%) | 91 (19%) | <0.001 | 103 (33%) | 83 (26%) | 0.068 |
| **Anti-hyperglycemic use** | 47 (10%) | 13 (3%) | <0.001 | 52 (17%) | 18 (6%) | <0.001 |

N(%) or mean (SD)

**Table 2**

Validation in the independent WHI-HT dataset of metabolites associations with future CHD discovered in the WHI-OS metabolites.

| Metabolite | WHI-HT Adjusted for matching[*] | | WHI-HT Adjusted for risk factors[†] | |
|---|---|---|---|---|
| | Odds Ratio[‡] | P-value[§] | Odds Ratio[‡] | P-value[§] |
| C34:2 hydroxy-PC | 1.83 (1.54, 2.17) | 5.4E-13 | 1.76 (1.47, 2.10) | 2.5E-10 |
| C36:4 hydroxy-PC | 1.66 (1.39, 1.99) | 2.4E-09 | 1.56 (1.30, 1.87) | 7.0E-07 |
| 15-HETE | 1.73 (1.44, 2.07) | 2.3E-10 | 1.65 (1.36, 1.99) | 5.6E-08 |
| 5-HETE | 1.71 (1.43, 2.04) | 3.0E-10 | 1.65 (1.37, 1.99) | 2.6E-08 |
| 11-HETE | 1.68 (1.41, 2.00) | 9.0E-10 | 1.64 (1.36, 1.97) | 4.3E-08 |
| 12-HETE | 1.64 (1.38, 1.95) | 3.9E-09 | | |
| glutamate | 1.64 (1.38, 1.95) | 5.5E-09 | 1.50 (1.25, 1.80) | 8.3E-06 |
| glutamine | 0.61 (0.50, 0.73) | 1.4E-08 | 0.67 (0.55, 0.82) | 2.5E-05 |
| asparagine | 0.75 (0.64, 0.88) | 4.2E-04 | | |
| CMP | 1.38 (1.17, 1.63) | 1.1E-04 | 1.33 (1.11, 1.59) | 1.4E-03 |
| uracil | 0.80 (0.68, 0.94) | 6.5E-03 | | |
| xanthosine | 0.80 (0.68, 0.95) | 7.5E-03 | | |
| glucose | 1.42 (1.19, 1.69) | 3.4E-05 | | |
| sucrose | 1.40 (1.18, 1.65) | 7.2E-05 | | |
| sorbitol | 1.28 (1.08, 1.51) | 3.4E-03 | | |
| 2-hydroxyglutarate | 1.45 (1.22, 1.72) | 1.0E-05 | | |
| fumarate/maleate | 1.22 (1.03, 1.44) | 2.1E-02 | | |
| isocitrate | 1.21 (1.02, 1.42) | 2.4E-02 | | |
| C50:0 TAG | 1.37 (1.16, 1.61) | 1.4E-04 | | |
| C50:1 TAG | 1.34 (1.14, 1.58) | 3.6E-04 | | |
| C52:0 TAG | 1.42 (1.20, 1.67) | 2.5E-05 | | |
| C52:1 TAG | 1.38 (1.17, 1.63) | 8.1E-05 | | |
| C52:2 TAG | 1.32 (1.12, 1.55) | 7.8E-04 | | |
| C54:1 TAG | 1.34 (1.14, 1.58) | 3.3E-04 | | |
| C54:2 TAG | 1.36 (1.15, 1.60) | 2.0E-04 | | |
| C54:3 TAG | 1.25 (1.06, 1.47) | 6.7E-03 | | |
| C56:2 TAG | 1.34 (1.14, 1.59) | 3.4E-04 | | |
| C56:3 TAG | 1.23 (1.05, 1.45) | 1.2E-02 | | |
| C34:0 DAG | 1.26 (1.06, 1.49) | 6.5E-03 | | |
| C34:1 DAG | 1.40 (1.18, 1.65) | 5.9E-05 | | |
| C36:1 DAG | 1.41 (1.19, 1.67) | 3.9E-05 | | |
| C36:2 DAG | 1.31 (1.11, 1.54) | 1.3E-03 | | |
| C40:10 PC | 0.82 (0.70, 0.97) | 1.8E-02 | | |

[*]
adjusted for baseline age, race/ethnicity, hysterectomy status, and enrollment window

†adjusted for baseline age, race/ethnicity, hysterectomy status, enrollment window, aspirin use, statin use, anti-hyperglycemic use, anti-hypertensive use, smoking, systolic blood pressure, diabetes, total and HDL cholesterol.

‡odds ratios are per 1 standard deviation of log transformed values

§Nominal p-values presented, cutoff for validation was nominal $p < 0.025$ and FDR adjusted p-value less than 0.05

**Table 3**

Validation of metabolites among men and women in PREDIMED

| Metabolite | All CVD* (N=980, 230 cases) | | CVD Men Only* (139 cases) | | CVD Women Only* (91 cases) | | Interaction by Sex P value |
|---|---|---|---|---|---|---|---|
| | Hazard Ratio (95%CI) | P value | Hazard Ratio (95%CI) | P value | Hazard Ratio (95%CI) | P value | |
| C34:2 Hydroxy-PC | 1.40 (1.15–1.70) | 0.0008 | 1.16 (0.90, 1.51) | 0.26 | 1.79 (1.30, 2.46) | 0.0004 | 0.013 |
| C36:4 Hydroxy-PC | 1.36 (1.12–1.66) | 0.0002 | 1.14 (0.86, 1.49) | 0.366 | 1.67 (1.22, 2.29) | 0.0015 | 0.026 |
| Glutamate | 1.48 (1.22–1.80) | 0.00009 | 1.39 (1.04, 1.84) | 0.024 | 1.64 (1.19, 2.24) | 0.0022 | 0.32 |
| Glutamine | 0.84 (0.64–1.11) | 0.22 | 0.83 (0.59, 1.19) | 0.32 | 0.79 (0.50, 1.26) | 0.33 | 0.65 |

*
adjusted for baseline age, sex, intervention group, systolic blood pressure, total cholesterol, high density lipoprotein cholesterol, diabetes status, smoking, statin use.