Metabolomic Effects of Hormone Therapy and Associations with Coronary Heart Disease among Postmenopausal Women

Running title: *Balasubramanian et al.; Metabolomic Effects of Hormone Therapy in Women*

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Abstract:

Background: In the Women's Health Initiative hormone therapy (WHI-HT) trials, treatment with oral conjugated equine estrogens and medroxyprogesterone acetate (CEE+MPA) resulted in increased risk of coronary heart disease (CHD), while oral conjugated equine estrogens alone (CEE) did not.

Methods: 481 metabolites were measured at baseline and at 1-year in 503 and 431 participants in the WHI CEE and CEE+MPA trials, respectively. The effects of randomized HT on the change in metabolite profiles at 1-year was evaluated in linear models adjusting for age, BMI, race, incident CHD, prevalent hypertension and diabetes. Metabolites with discordant effects by HT type were evaluated for association with incident CHD in 944 participants (472 CHD cases) in the WHI Observational Study (WHI-OS), with replication in an independent cohort of 980 men and women at high risk for cardiovascular disease.

Results: HT effects on the metabolome were profound; 62% of metabolites significantly changed with randomized CEE and 52% with CEE+MPA (FDR adjusted p value < 0.05) in multivariable models. Concerted increases in abundance were seen within various metabolite classes including triacylglycerols (TAG), phosphatidylethanolamines and phosphatidylcholines (PC); decreases in abundance was observed for acylcarnitines, lysophosphatidylcholines, quaternary amines and cholesteryl/cholesteryl esters. Twelve metabolites had discordant effects by HT type and were associated with incident CHD in the WHI-OS; a metabolite score estimated in a LASSO regression was associated with CHD risk with an odds ratio of 1.47 per SD increase (95% CI: 1.27-1.70, $p<10^{-6}$). The findings of a subset of four metabolites including C58:11 TAG, C54:9 TAG, C36:1 PC and sucrose replicated in an independent dataset of 980 participants.

Conclusions: Randomized treatment with oral HT resulted in large metabolome shifts. Discordant metabolite effects between HT regimens may partially mediate the differences in CHD risk between the two WHI-HT trials.

Key words: cardiovascular disease; women; hormones; metabolome; hormonal therapy; metabolomics; women's health

Nonstandard Abbreviations and Acronyms
WHI-HT: Women's Health Initiative hormone therapy trials
WHI-OS: Women's Health Initiative Observational Study
WHI: Women's Health Initiative
PREDIMED: Prevención con Dieta Mediterránea trial
HT: Hormone therapy
CEE+MPA: conjugated equine estrogens and medroxyprogesterone acetate
CEE: conjugated equine estrogens alone
CHD: Coronary heart disease
CVD: Cardiovascular disease
MI: Myocardial infarction
FDR: False discovery rate
SD: standard deviation
OR: Odds Ratio
MSEA: Metabolite Set Enrichment Analysis
BCAA: branched-chain amino acids
DAG or MAG: Di(Mono)acyl glycerol
TAG: triacylglycerol
GP: glycerophopholipid
LPC or LPC(P): Lysophosphatidyl choline or Lysophosphatidyl choline plasmalogen
LPE: Lysophosphatidyl ethanolamine
PC: Phosphatidyl choline
PC [P]: Phosphatidyl choline plasmalogen
PE: Phosphatidyl ethanolamine
PE[P]: Phosphatidyl ethanolamine plasmalogen
C&CE: Cholestyrl or Cholesteryl Ester
SL: Other sphingolipid
SM: sphingomyelin
FA: Fatty acid
AC: Acylcarnitine
AA: Amino acid
BA: Bile acid
PU&PY: purines and pyrimidines
QA: Quaternary amine
IND: Indole and indole derivatives
PYR: pyridines and derivatives
OA: Other organic acid
CARB: Carbohydrates and conjugates
BZ: Benzene and derivatives
OTH: other.
Health ABC study: Health, Aging, and Body Composition Study
HAI: Healthy Aging Index

Introduction

In the Women's Health Initiative (WHI) hormone therapy (HT) trials, treatment with oral conjugated equine estrogens and medroxyprogesterone acetate (CEE+MPA) resulted in a 29% increased risk of coronary heart disease (CHD), while treatment with conjugated equine estrogens alone (CEE) was associated with a non-significant 9% reduction in CHD [1-3]. During the randomized treatment, CEE+MPA also resulted in increased risk of invasive breast cancer, stroke, pulmonary embolism, dementia (in women aged ≥65 years), gallbladder disease, and urinary incontinence, while it reduced hip fractures, colorectal cancer, and diabetes [3]. In the CEE alone trial, higher risk of stroke, reduced risk of hip fracture, and a trend toward decreased risk of breast cancer resulted with randomized therapy [3]. Differences in the effects of these two HT regimens on metabolomic profiles is largely unknown and such differences might mediate differences in outcomes.

Metabolites in body fluids reflect multiple biochemical processes relevant to health and disease [4]. Recent advances in metabolite profiling techniques (metabolomics) have enhanced our ability to measure a full profile of small-molecule metabolites, thus, providing a comprehensive picture of an individual's metabolic status. Metabolite measures closely reflect the underlying molecular pathways governing various disease processes and thus enhance our understanding of the etiology of complex disorders. Differences in HT effects on metabolomic profiles and their association with CHD may shed light on the determinants of the observed differences in CHD and other disease outcomes between the CEE+MPA and CEE clinical trials in the WHI.

We sought to characterize the effects of randomized HT on the metabolome and evaluate whether these changes might explain the differences in CHD outcomes between the two trials. We utilized two independent datasets from a metabolomics of CHD case control study nested

within the WHI [5]. A total of 934 participants in the WHI-HT had metabolomic profiles measured at baseline and at year 1 following randomization and had no prior cardiovascular disease (CVD); metabolite changes were compared between the active HT vs placebo groups. Metabolites that demonstrated discordant effects of CEE and CEE+MPA treatments were further evaluated in a subset of 944 participants in the WHI Observational Study (OS) for association with incident CHD risk, and associations replicated among 647 participants randomized to the placebo arms of the WHI-HT trials. The metabolite-CHD associations identified in the WHI-OS were also tested for replicability in a metabolomics dataset nested within the Prevención con Dieta Mediterránea (PREDIMED) trial.

Methods

All participants in the WHI and PREDIMED cohorts provided written informed consent. This study was approved by the Institutional Review Board of Mass General Brigham. The overview of the study design involving data from the WHI and PREDIMED cohorts is shown in Figure 1. The characteristics of the WHI participants are shown in Tables 1-2. The details of the materials and methods are described in the Data Supplement.

The data included in this study are subject to human subject protections and cannot be made freely available. Metabolite data from the WHI analyzed in this study are available from the dbGaP database under dbGaP accession no. phs001334.v1.p3.22. All other WHI data described in the manuscript, code book, and analytic code is available by request/research proposal at Women's Health Initiative (www.whi.org).

Results

Study population

Metabolite values at baseline and 1 year were measured for 934 women, including 503 in the CEE trial (240 active, 263 placebo) and 431 in the CEE+MPA trial (235 active, 196 placebo). CHD case/control distributions were similar in both trials (by design), as were age, BMI, systolic blood pressure distributions and the prevalence of current smokers (Table 1). The CEE trial had a higher proportion of African Americans and higher prevalence of diabetes than the CEE+MPA (Table 1).

Hormone therapy effects on the metabolome

HT effects on the metabolome were profound. In the CEE trial, 298 of 481 (62%) metabolites were significantly altered (FDR adjusted p value < 0.05) by treatment after adjustment for age, BMI, CHD case/control status, race, prevalent diabetes and hypertension with 162 metabolites decreasing and 136 increasing with CEE treatment (Figure 2, Supplemental Table 1). In the CEE+MPA trial, 249 of 481 (52%) metabolites were significantly altered (FDR adjusted p value < 0.05) by treatment, after adjustment for the same set of confounders, with 132 decreasing and 117 increasing with CEE+MPA treatment (Figure 3, Supplemental Table 2). There was no evidence of an interaction between the effects of each HT with baseline age (see Data Supplement page 7) [6].

HT resulted in a large magnitude of change in many metabolites, including 5 with greater than 1 standard deviation (SD) change with CEE and 2 for CEE+MPA (Figures 2-3). Metabolites with a decrease of more than 1 SD included betaine, C18:0 carnitine, C18:0 LPC-A, C18:0 LPC with CEE, and betaine and C18:0 LPC-A with CEE+MPA. The average increase in levels of C38:6 PE with CEE was greater than 1 SD.

Metabolites that were altered with active HT were from many metabolite classes (Supplemental Figures 1-3). Evidence of concerted changes due to active HT relative to placebo within each of 24 metabolite classes was evaluated by MSEA, where the threshold for statistical significance was an FDR adjusted p value less than 0.05. As shown in Figure 4, CEE treatment resulted in positive enrichment of metabolites belong to TAG, DAG/MAG, PC and PE and significant negative enrichment of metabolites belonging to LPC/LPC(P), AC, QA, SL and C&CE classes in MSEA analyses. Similar changes were seen with CEE+MPA treatment, with additional negative enrichment of AA, and lack of MSEA enrichment in SL.

When class changes in abundance were examined, between 45%-83% of the metabolites belonging to the lipid classes TAG, DAG/MAG, PC and PE increased in abundance with CEE relative to placebo; conversely, between 57%-87% of all metabolites within the classes of LPC or LPC(P), AC, QA, SL and C&CE decreased in abundance with CEE relative to placebo (Supplemental Figure 1). Similar trends were seen with CEE+MPA treatment (Supplemental Figure 2), but with smaller proportions of significant changes within several metabolite classes.

TAGs demonstrated large changes with HT, where 50 of the 71 TAGs measured (70%) were altered with CEE, and 40 by CEE+MPA (56%). With CEE, levels increased in 46 TAGs, with a mean change of 0.31 SD units; levels decreased in 4 TAGs with a mean change of -0.29 SD units. With CEE+MPA, 37 TAGs increased with a mean change of 0.28 SD and 3 decreased with a mean change of -0.24 SD units (Supplemental Figure 3). TAGs that changed due to active HT included those with carbon chain lengths between 42-54 and with fewer than 5 double bonds; as well as TAGs with longer carbon chain lengths between 56-60, but with high degree of unsaturation (5-12 double bonds).

Discordant metabolome effects between hormone therapy regimens

While there was considerable overlap in the metabolite changes between CEE and CEE+MPA, there was also evidence specific effects by HT type (Supplemental Figures 1-2). Among 71 TAGs, 17% significantly changed with CEE but not with CEE+MPA; in contrast, only 2.8% of TAGs significantly changed with CEE+MPA but not with CEE. Of 26 acylcarnitines, 27% significantly changed in abundance with CEE but not with CEE+MPA; in contrast, no changes among acylcarnitines were observed that were exclusive to CEE+MPA. Similarly, among 34 PCs, 24% significantly changed with CEE but not with CEE+MPA; however, none changed exclusively with CEE+MPA. In general, the number of metabolites significantly changing due to active HT was larger in the CEE trial than in the CEE+MPA trial. A resampling analysis confirmed that the observation of a fewer number of changes in the metabolome by CEE+MPA when compared to CEE was not entirely driven by sample size differences between trials (see Supplement page 8).

Tables 3-4 present the 22 metabolites with evidence of discordant effects by HT type – for each of these metabolites, a test of the equality of the CEE and CEE+MPA effects was rejected at a p value threshold of 0.05. Lysine was significantly altered by both CEE and CEE+MPA, but in opposite directions with an increase in levels with CEE and a decrease in levels due to CEE+MPA. Nineteen metabolites were changed by CEE but not CEE+MPA. Increased levels CEE relative to placebo were observed for 7 TAGs (C54:9, C54:7, C56:9, C56:8, C56:7, C56:6, C58:11), 2 PC plasmalogens (C38:7, C38:6), and glycocholate. CEE treatment resulted in decreased abundance for C36:1 PC plasmalogen, C18:0 SM, 2 carnitines (C7 and C18:1-OH), creatinine, fumarate/maleate, malate, sucrose and hydroxyphenyacetate (Table 4). Only 2 metabolites were changed by CEE+MPA but not CEE; 7-methyl guanine

increased with CEE+MPA, whereas levels of NMMA decreased with CEE+MPA (Table 4). The missing value distribution and coefficient of variation estimates for this subset of 22 metabolites is shown in Supplemental Table 3.

Associations of metabolites with discordant effects between HT regimens with incident CHD risk

The 22 discordant metabolites were evaluated for association with incident CHD risk in an independent WHI-OS dataset with 472 incident CHD cases (median time to event of 5.8 years) and 472 frequency-matched controls (5). Table 2 shows the characteristics of the participants in the WHI-OS dataset. Twelve metabolites were associated with CHD risk (FDR adjusted p value < 0.05), after adjusting for matching and traditional CHD risk factors including age, race, hysterectomy status, WHI enrollment time period, smoking, systolic blood pressure, diabetes, total and HDL cholesterol, use of aspirin, anti-hyperglycemic drugs and anti-hypertensive drugs (Table 5, Figure 5). All twelve metabolites were altered in the CHD protective direction by CEE treatment, including 7 TAGs (C54:9, C 54:7, C 56:9, C56:8, C56:7, C 56:6, C 58:11), C36:1 PC plasmalogen, lysine, fumarate/maleate, sucrose and hydroxyphenylacetate. Lysine was significantly altered in the direction of increased CHD risk by CEE+MPA; the remaining 11 metabolites were not significantly changed by CEE+MPA (Table 5, Figure 5).

From the 12 metabolites, a sparse metabolite fingerprint associated with CHD risk was identified in a Lasso logistic regression model that included 2 TAGS (C54:9, C56:9), lysine, fumarate/maleate, sucrose and hydroxyphenylacetate (Supplemental Table 4). The regression coefficients estimated in the LASSO regression were used to calculate a metabolite score. The metabolite score was significantly associated with incident CHD risk ($p < 10^{-6}$) after adjustment for matching and CHD risk factors, with an odds ratio (OR) of 1.47 (95% CI: 1.27-1.70) per SD

increase in the score. There was no evidence of an interaction of metabolite score with age in predicting CHD risk (p=0.20).

As a validation, the metabolite score at baseline was evaluated for association with incident CHD risk in 1362 participants (681 incident CHD cases) in the active and placebo arms of the two WHI-HT trials. The odds ratio (OR) for CHD per SD increase in the metabolite score was 1.23 (95% CI: 1.09 – 1.38, $p < 0.001$), after adjustment for traditional CHD risk factors. When the analysis was restricted to 647 participants (322 incident CHD cases) in the placebo arms of the two WHI-HT trials, the odds ratio (OR) for CHD per SD increase in the metabolite score was 1.19 (95% CI: 1.00 – 1.42, $p = 0.06$), after adjustment for traditional CHD risk factors.

The metabolite-CHD associations identified in the WHI-OS were tested for replicability in the PREDIMED study. 11 of the 12 metabolites (with the exception of hydroxyphenylacetate) were available for evaluation in the PREDIMED study. Each metabolite was evaluated for association with a combined CVD endpoint of incident MI, stroke, and CHD death (224 cases, mean follow up of 4.8 years). Baseline characteristics of the PREDIMED study are shown in Supplemental Table 5. For the combined CVD outcome in men and women, 3 metabolites replicated in risk factor adjusted models (p value < 0.05), including fumarate/maleate (OR=1.37, 95% CI: 1.15-1.62), sucrose (OR=1.33, 95% CI: 1.13-1.56) and C58:11 TAG (OR=0.84, 95% CI: 0.71-0.99); C36:1 PC was marginally significant (p value < 0.1), with an OR of 1.17 (95% CI: 0.98-1.40) (Table 6). Additionally, C58:11 TAG showed evidence of an interaction effect with sex (p-interaction = 0.03). Among females (n=530), C58:11 TAG was inversely associated with CVD risk (p=0.02, OR=0.71, 95% CI:0.54-0.94) (Table 6).

Effects of hormone therapy on triacylglycerols (TAGs) and incident CHD risk

Of the 50 TAGs changing in abundance with CEE, 22 were altered in the CHD protective

direction after adjustment for all confounders – all were observed to significantly increase with CEE and had inverse associations with CHD risk in the WHI-OS (FDR adjusted p value < 0.05). None were altered in the direction of increased CHD risk with CEE (Supplemental Table 6). Of the 40 TAGs altered with CEE+MPA, 15 were in the CHD protective direction; none were altered in the direction of increased CHD risk with CEE+MPA (Supplemental Table 7). TAGs with a higher degree of unsaturation were more likely to be changed in the CHD protective direction by CEE (OR=1.95 corresponding to a unit increase in unsaturation, 95% CI: 1.42-2.90, p=0.0002) (Supplemental Figure 4); no significant effect was observed for carbon chain length (p =0.12). Both degree of unsaturation and carbon chain length were not predictors of direction of change due to CEE+MPA (p > 0.15).

Discussion

Our study evaluated the impact of randomized HT treatment on metabolite levels and found substantial changes with both CEE and CEE+MPA compared to placebo. Overall, 62% of metabolites significantly changed with randomized CEE and 52% significantly changed with CEE+MPA at 1 year in the active treatment groups compared with placebo (FDR adjusted p value < 0.05), after adjustment for metabolite score at baseline, age, BMI, CHD case/control status, race, prevalent diabetes and hypertension. These changes were seen across a wide range of metabolite classes including TAGs, AAs, PCs and ACs.

Detailed data on the impact of HT on the metabolome have been limited. In a recent cross-sectional analysis in the Cancer Prevention II Nutrition Cohort, a large number of metabolites were observed to have differential abundance between HT users and non-users, with 35% of metabolites significantly associated with estrogen-only use and 28% associated with

estrogen plus progestin use [7]. In particular, levels of numerous lipids, ACs and AAs differed between HT users and non-users. However, this study was cross-sectional, not randomized, and had only a single metabolomic measure.

In the WHI-HT trials, treatment with CEE+MPA resulted in increased risk of CHD; however, these increased risks were not observed in the CEE arm [1, 2]. Twenty-two metabolites had significantly discordant effects between CEE and CEE+MPA, of which 12 were also associated with risk of incident CHD in an independent dataset of 944 participants nested within the WHI-OS cohort. Of these, 4 metabolites were associated with total CVD risk in the PREDIMED study in risk factor adjusted models, with an additional metabolite (C54:9 TAG) that replicated only within the subgroup of women.

A CHD metabolite score comprised of a sparse set of six discordant metabolites was associated with a 47% increased risk of CHD per SD in the WHI-OS, and was replicated in a separate set of women in the WHI-HT placebo arms. Consistent with the WHI-HT findings of no observed CHD risk with CEE, all six metabolites were changed in the CHD protective direction by CEE and 1 metabolite (lysine) was changed in the direction of increased CHD risk by CEE+MPA.

HT is known to cause increases in triglycerides and 2 TAGs (C56:9, C54:9) were included in our CHD metabolite risk score. In our study, 65% of TAGS increased with CEE and 52% increased with CEE+MPA. When we compared the effect of the two HT regimens, we identified 7 TAGs (C54:9, C54:7, C56:9, C56:8, C56:7, C56:6, C58:11) that were increased with CEE but not with CEE+MPA (Table 4). Higher levels of all 7 TAGs were associated with reduced risk of CHD after adjustment for a full set of risk factors (FDR adjusted p value < 0.05) (Figure 5). Additionally, C54:9 TAG had a significant interaction effect with sex in the

PREDIMED study, with a significant inverse association with CVD observed only in the subgroup of women (Table 6). In contrast, another non-overlapping set of TAGs and DAGs with a lower double bond content (0-3), were associated with CHD in our prior work in the WHI [5].

Changes in amino acid changes were prominent with HT. The amino acid lysine was the strongest component of the CHD metabolite score, with higher levels associated with reduced CHD risk. Lysine levels were increased with CEE and decreased with CEE+MPA (FDR adjusted p value < 0.05). Prior literature on the association of branched-chain amino acids (BCAA) including leucine, lysine and valine with CHD is complex. Several cohort studies have shown that higher levels of BCAAs are associated with higher risk of CHD and other cardiometabolic disorders [8-11]. In contrast, studies in human and animal models have demonstrated that higher dietary intake of BCAAs are associated with cardiovascular benefits [12, 13]. Several previous studies have also found sex differences in the relationship between BCAAs and insulin resistance with stronger evidence for the relationship in men [14, 15]. Thus, changes in amino acids with CEE versus CEE+MPA may partially explain the observed differences in CHD risk.

Fumarate or fumaric acid is a dicarboxylic acid and a pre-cursor to L-malate in the Kreb's tricarboxylic acid (TCA) cycle. Recent literature points to fumarate as a carcinogenic metabolite[16]. In our study, higher levels of fumarate were associated with increased CHD and fumarate levels were lowered by CEE in the CHD protective direction, with no corresponding change for CEE+MPA. High levels of fumarate were also associated with increased CVD risk in the PREDIMED study (Table 6).

The CHD metabolite score also included sucrose and hydroxyphenylacetate, where higher levels of each was associated with increased CHD risk. It is well established that added sugars such as sucrose in diet are associated with insulin resistance and increased CVD risk[17].

Hydroxyphenylacetate is a phenol that was significantly associated with long-term cardiovascular mortality among 291 African American men in the Health, Aging, and Body Composition (Health ABC) study (univariable hazard ratio of 1.39 (95% CI: 1.12-1.74) per SD increase). Moreover, in the Health ABC study, hydroxyphenylacetate was observed to be significantly increased among participants in the "unhealthy" category of the Healthy Aging Index (HAI) relative to those in the optimal HAI category[18].

This study has several strengths including analysis of two randomized HT regimens in well-characterized populations and a broad metabolomics platform. However, this study is limited by the two specific HT regimens used, at specific doses. It is unclear how route of administration (i.e., oral vs transdermal), formulation of estrogen or progestin, and dose might affect the observed associations. We cannot rule out that some changes in the effects of metabolites with type of HT may have been due to underlying differences in the populations of women with and without hysterectomy. Moreover, the participants in the WHI-HT trials were an average of 66-67 years of age at the time of randomization and relatively distant from the average age of menopause, but age at baseline did not modify the results.

In summary, HT treatment resulted in significant changes in metabolites, across a wide range of metabolite classes. While a majority of metabolite changes were consistent between both treatment arms, there was evidence of fewer changes in metabolite profiles with CEE+MPA than CEE alone. In terms of associations with CHD risk, discordant effects by HT type on metabolites generally favored CEE alone. Differences in the effects of HT on metabolomic profiles may partially mediate the observed disease associations in the WHI-HT trials.

Future research in younger cohorts and with other formulations and doses of hormone therapy would further shed light on the HT-related effects on the metabolome and potential

implications for CHD risk. Differential effects by HT type were also observed for invasive breast cancer, stroke and other chronic conditions. Future research could identify HT-related metabolomic changes that may partially explain the observed disparities in disease associations between HT regimens.

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Data availability: Metabolite data analyzed in this study have been deposited in and are available from the dbGaP database under dbGaP accession no. phs001334.v1.p3.22. All other WHI data described in the manuscript, code book, and analytic code is available by request/research proposal at Women's Health Initiative (www.whi.org).
A list of WHI investigators is available online at https://www.whi.org/researchers/Documents%20%20Write%20a%20Paper/WHI%20Investigator%20Short%20List.pdf.

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Table 1: Baseline characteristics of participants in the Women's Health Initiative Observational Study (WHI-OS) and the Women's Health Initiative Hormone Therapy (WHI-HT) Trials .

Characteristic	WHI-HT [CEE versus Placebo]			WHI-HT [CEE+MPA versus Placebo]		
	Placebo (n=263)	CEE (n=240)	P value*	Placebo (n=196)	CEE+MPA (n=235)	P value*
Age (years) [Mean (SD)]	66.09 (6.95)	67.18 (6.47)	0.07	66.41 (7.21)	66.54 (7.36)	0.86
BMI (kg/m^2) [Mean (SD)]	29.90 (5.95)	30.14 (5.64)	0.64	28.69 (5.88)	28.40 (5.87)	0.61
Systolic blood pressure [Mean (SD)]	134.73 (18.78)	134.13 (17.59)	0.71	132.49 (17.79)	131.17 (19.09)	0.46
Race (%)			0.0005			0.05
• Black or African-American	20.15%	10.83%		6.12%	6.38%	
• Hispanic/Latino	4.18%	0.83%		0%	2.98%	
• Other	0.76%	0%		2.55%	0.85%	
• White (not of Hispanic origin)	74.14%	88.33%		91.33%	89.79%	
Current Smoking (%)	15.21%	13.33%	0.31	13.27%	14.89%	0.75
Prevalent Diabetes (%)	19.01%	15.83%	0.41	12.76%	12.77%	1.00
Prevalent Hypertension (%)	49.81%	51.25%	0.93	43.37%	40.43%	0.46
Incident deaths (%)						
• All-cause	50.19%	49.58%	0.94	50%	47.23%	0.63
• CVD	30.80%	26.67%	0.34	22.96%	24.26%	0.82
Incident CHD (%)	48.67%	50.83%	0.68	49.49%	50.64%	0.85

* P values comparing active treatment to placebo for continuous variables were from 2 sample t tests. P values for categorical variables were from Chi square tests with Monte Carlo simulated p values.

Table 2: Baseline characteristics of 944 participants in the Women's Health Initiative Observational Study (WHI-OS).

	WHI-OS (N=944)		
	Cases N = 472	**Controls N = 472**	**p-value**
Age, years	68 (7)	67 (7)	0.51
Race			1.00
White	348 (73%)	348 (73%)	
Black	69 (15%)	69 (15%)	
Other	55 (12%)	55 (12%)	
Systolic blood pressure, mmHg	135 (19)	130 (18)	<0.001
Diabetes	78 (17%)	25 (5%)	<0.001
BMI, m^2/kg	29 (6)	27 (6)	0.001
Total cholesterol, mg/dL	231 (47)	232 (47)	0.51
HDL cholesterol, mg/dL	51 (16)	57 (17)	<0.001
Smoking status,			0.016
Current	48 (10%)	28 (6%)	
Former	209 (44%)	243 (51%)	
Never	215 (46%)	201 (43%)	
Aspirin use	116 (25%)	98 (21%)	0.16
Statin use	42 (9%)	39 (8%)	0.72
Anti-hypertensive use	144 (31%)	91 (19%)	<0.001
Anti-hyperglycemic use	47 (10%)	13 (3%)	<0.001

N(%) or mean (SD)

Table 3: Direction of change among metabolites with discordant HT effects, by treatment arm in the CEE and CEE+MPA WHI HT trials. The numbers in bold correspond to metabolites classified as having discordant HT effects.

		CEE + MPA versus Placebo			
		Decrease	**No evidence for change**	**Increase**	**Total**
CEE versus Placebo	Decrease	0	9	0	9
	No evidence for change	1	0	1	2
	Increase	1	10	0	11
	Total	2	19	1	22

Table 4: Twenty-two metabolites with differential effects when comparing CEE alone to CEE+MPA.

		CEE versus Placebo			CEE+MPA versus Placebo			
Metabolite*	**HT†**	**p value**	**FDR adjusted p value**	**CEE effect‡**	**p value**	**FDR adjusted p value**	**CEE+MPA effect‡**	**Difference in HT effect§**
C36:1 PC plasmalogen	CEE	1.84E-05	2.55E-05	-0.31 (-0.44, -0.17)	2.29E-01	1.77E-01	-0.10 (-0.25, 0.06)	-0.21 (-0.42, 0.00)
C38:7 PC plasmalogen	CEE	3.61E-03	3.15E-03	0.19 (0.06, 0.31)	7.62E-01	4.46E-01	-0.02 (-0.16, 0.12)	0.21 (0.02, 0.40)
C38:6 PC plasmalogen-A	CEE	9.16E-03	7.23E-03	0.18 (0.05, 0.32)	4.49E-01	3.00E-01	-0.06 (-0.21, 0.09)	0.24 (0.04, 0.45)
C18:0 SM	CEE	4.58E-08	8.89E-08	-0.41 (-0.55, -0.27)	6.93E-02	6.82E-02	-0.15 (-0.31, 0.01)	-0.26 (-0.48, -0.05)
C54:9 TAG	CEE	9.90E-04	9.60E-04	0.23 (0.09, 0.36)	6.74E-01	4.05E-01	-0.04 (-0.20, 0.13)	0.26 (0.05, 0.47)
C54:7 TAG	CEE	2.80E-02	1.96E-02	0.15 (0.02, 0.29)	3.39E-01	2.37E-01	-0.07 (-0.22, 0.08)	0.23 (0.02, 0.43)
C56:9 TAG	CEE	1.70E-04	1.94E-04	0.27 (0.13, 0.40)	9.29E-01	5.10E-01	-0.01 (-0.16, 0.14)	0.27 (0.07, 0.48)
C56:8 TAG	CEE	9.43E-06	1.36E-05	0.31 (0.18, 0.45)	3.12E-01	2.24E-01	0.08 (-0.07, 0.23)	0.24 (0.03, 0.44)
C56:7 TAG	CEE	4.51E-04	4.75E-04	0.24 (0.11, 0.37)	7.00E-01	4.18E-01	0.03 (-0.12, 0.18)	0.21 (0.01, 0.41)
C56:6 TAG	CEE	9.47E-05	1.16E-04	0.26 (0.13, 0.39)	4.37E-01	2.94E-01	0.05 (-0.08, 0.19)	0.21 (0.02, 0.40)
C58:11 TAG	CEE	1.88E-06	3.03E-06	0.31 (0.18, 0.44)	6.97E-01	4.17E-01	0.03 (-0.13, 0.19)	0.28 (0.08, 0.48)

7-methylguanine	CEE+MPA	3.54E-01	1.78E-01	-0.07 (-0.21, 0.07)	1.55E-02	1.90E-02	0.20 (0.04, 0.36)	-0.27 (-0.48, -0.05)
C7 carnitine	CEE	5.77E-06	8.59E-06	-0.35 (-0.50, -0.20)	1.32E-01	1.14E-01	-0.12 (-0.27, 0.03)	-0.23 (-0.44, -0.02)
C18:1-OH carnitine	CEE	7.22E-03	5.98E-03	-0.22 (-0.38, -0.06)	5.15E-01	3.37E-01	0.05 (-0.11, 0.22)	-0.27 (-0.50, -0.05)
creatinine	CEE	1.90E-03	1.73E-03	-0.19 (-0.31, -0.07)	9.40E-01	5.13E-01	0.00 (-0.11, 0.12)	-0.20 (-0.36, -0.03)
glycocholate	CEE	2.77E-02	1.94E-02	0.21 (0.02, 0.39)	1.30E-01	1.13E-01	-0.14 (-0.32, 0.04)	0.35 (0.09, 0.60)
lysine	CEE CEE+MPA	4.29E-05	5.59E-05	0.31 (0.16, 0.46)	3.15E-02	3.57E-02	-0.18 (-0.35,-0.02)	0.50 (0.27, 0.72)
NMMA	CEE+MPA	1.28E-01	7.43E-02	0.11 (-0.03, 0.26)	3.81E-02	4.20E-02	-0.18 (-0.35,-0.01)	0.29 (0.07, 0.52)
fumarate/maleate	CEE	1.85E-06	3.00E-06	-0.34 (-0.48, -0.20)	6.59E-02	6.54E-02	0.15 (-0.01, 0.30)	-0.49 (-0.70, -0.28)
malate	CEE	9.33E-03	7.33E-03	-0.18 (-0.31, -0.04)	1.26E-01	1.09E-01	0.12 (-0.03, 0.28)	-0.30 (-0.51, -0.09)
sucrose	CEE	1.48E-03	1.38E-03	-0.25 (-0.41, -0.10)	2.54E-01	1.91E-01	0.09 (-0.07,0.26)	-0.35 (-0.57, -0.12)
hydroxyphenylacetate	CEE	2.49E-02	1.76E-02	-0.17 (-0.31, -0.02)	2.93E-01	2.13E-01	0.08 (-0.07, 0.23)	-0.25 (-0.46, -0.04)

*TAG – Triacylglycerol; PC – phophatidylcholine; SM – sphingomyelin; NMMA – N-methylmalonamic acid
† HT denotes the treatment arm (relative to placebo) where a significant difference in metabolite levels was observed.
‡Treatment effect is the average difference due to active treatment (active treatment minus placebo) at year 1, on natural logarithm transformed, standardized units.
§Difference in HT effect is calculated as the average change due to CEE minus the average change due to CEE+MPA

Table 5: Metabolites with discordant treatment effects in the WHI-HT and their associated incident CHD risk profile in the Women's Health Initiative Observational Study (n=944).

Metabolite	Direction of change with CEE	Direction of change with CEE+MPA*	Incident CHD† association OR (95% CI) p value	HT effect with respect to CHD	
C36:1 PC plasmalogen	Decrease	No change	1.16 (1.01, 1.35) p=0.04	CEE protective	CEE+MPA no change
C54:9 TAG	Increase	No change	0.79 (0.68, 0.92) p=0.002	CEE protective	CEE+MPA no change
C54:7 TAG	Increase	No change	0.77 (0.67, 0.90) p=0.001	CEE protective	CEE+MPA no change
C56:9 TAG	Increase	No change	0.79 (0.69, 0.91) p=0.001	CEE protective	CEE+MPA no change
C56:8 TAG	Increase	No change	0.81 (0.70, 0.93) p=0.002	CEE protective	CEE+MPA no change
C56:7 TAG	Increase	No change	0.79 (0.69, 0.91) p=0.001	CEE protective	CEE+MPA no change
C56:6 TAG	Increase	No change	0.82 (0.71, 0.94) p=0.005	CEE protective	CEE+MPA no change
C58:11 TAG	Increase	No change	0.82 (0.71, 0.95) p=0.007	CEE protective	CEE+MPA no change
lysine	Increase	Decrease	0.80 (0.69, 0.92) p=0.002	CEE protective	CEE+MPA increased risk
fumarate/maleate	Decrease	No change	1.17 (1.02, 1.35) p=0.03	CEE protective	CEE+MPA no change
sucrose	Decrease	No change	1.16 (1.01, 1.33) p=0.03	CEE protective	CEE+MPA no change
hydroxyphenylacetate	Decrease	No change	1.16 (1.01, 1.34) p=0.03	CEE protective	CEE+MPA no change

* 'No change' when a hypothesis test comparing metabolite abundance in the active HT arm to the placebo arm failed to reject the null hypothesis (FDR adjusted p value > 0.05).

† CHD model in the WHI-OS adjusted for age, hysterectomy, time period of enrollment, ethnicity, hypertension treatment, diabetes treatment, aspirin, statin use, systolic blood pressure, total cholesterol, HDL cholesterol, diabetes, smoking. All 12 metabolites met a threshold of FDR adjusted p value < 0.05 for association with CHD risk.

Table 6: Association of 11 metabolites with incident total CVD among men and women in PREDIMED (n=980).

	All CVD* (N=980, 224 cases)		CVD Men Only* (N=450, 136 cases)		CVD Women Only* (N=530, 88 cases)		Interaction by Sex
Metabolite	Odds Ratio (95%CI)	P value	Odds Ratio (95%CI)	P value	Odds Ratio (95%CI)	P value	P value
Fumarate/maleate	1.37 (1.15-1.62)	0.00035	1.39 (1.11 – 1.75)	0.006	1.35 (1.03 – 1.77)	0.03	0.86
Sucrose	1.33 (1.13-1.56)	0.00076	1.37 (1.08 – 1.75)	0.01	1.27 (1.01 – 1.61)	0.04	0.93
C58:11 TAG	0.84 (0.71 – 0.99)	0.035	0.86 (0.69 – 1.07)	0.17	0.79 (0.61 – 1.02)	0.07	0.43
C36:1 PC	1.17 (0.98 – 1.40)	0.079	1.09 (0.86 – 1.38)	0.48	1.27 (0.96 – 1.69)	0.09	0.91
C56:6 TAG	0.89 (0.76 – 1.05)	0.17	0.90 (0.72 – 1.13)	0.38	0.86 (0.68 – 1.10)	0.23	0.85
C56:9 TAG	0.87 (0.74 – 1.03)	0.11	0.89 (0.72 – 1.10)	0.29	0.84 (0.64 – 1.08)	0.18	0.59
C54:9 TAG	0.90 (0.78 – 1.05)	0.19	0.99 (0.82 – 1.19)	0.89	0.71 (0.54 – 0.94)	0.02	0.03
C56:8 TAG	0.92 (0.78 – 1.09)	0.32	0.93 (0.75 – 1.15)	0.50	0.89 (0.68 – 1.15)	0.37	0.72
C56:7 TAG	0.95 (0.80 – 1.13)	0.57	0.94 (0.75 – 1.17)	0.59	0.94 (0.72 – 1.23)	0.65	0.82
lysine	1.05 (0.89 – 1.23)	0.55	1.00 (0.81 – 1.23)	0.96	1.13 (0.87 – 1.48)	0.35	0.47
C54:7 TAG	0.95 (0.80 – 1.13)	0.58	0.94 (0.76 – 1.17)	0.59	0.93 (0.70 – 1.24)	0.63	0.91

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

Figure Legends:

Figure 1: Study design evaluating metabolomic changes with HT (Phase 1) and with incident CHD risk (Phase 2).

Figure 2: Mean change in relative abundance due to CEE when compared to placebo (Beta E values) versus statistical significance. Mean change due to HT (X axis) and statistical significance (Y axis) estimated in linear models with metabolite levels at Year 1 on the natural logarithm scale as the outcome (Y) and treatment indicator (CEE versus placebo) as the exposure, while adjusting for metabolite levels at baseline, age, BMI, CHD case control status, race, prevalent diabetes and hypertension. The following acronyms are used to define metabolite classes: DAG or MAG: Di(Mono)acyl glycerol, TAG: triacylglycerol, GP: glycerophopholipid, LPC or LPC(P): Lysophosphatidyl choline or Lysophosphatidyl choline plasmalogen; LPE: Lysophosphatidyl ethanolamine; PC: Phosphatidyl choline; PC [P]: Phosphatidyl choline plasmalogen; PE: Phosphatidyl ethanolamine; PE[P]: Phosphatidyl ethanolamine plasmalogen; C&CE: Cholestyrl or Cholesteryl Ester; SL: Other sphingolipid; SM: sphingomyelin; FA: Fatty acid; AC: Acylcarnitine; AA: Amino acid; BA: Bile acid; PU&PY: purines and pyrimidines; QA: Quaternary amine; IND: Indole and indole derivatives; PYD: pyridines and derivatives; OA: Other organic acid; CARB: Carbohydrates and conjugates; BZ: Benzene and derivatives; OTH: other.

Figure 3: Mean change in relative abundance due to CEE+MPA when compared to placebo (Beta EP Values) versus statistical significance. Mean change due to HT (X axis) and statistical significance (Y axis) estimated in linear models with metabolite levels at Year 1 on the natural

logarithm scale as the outcome (Y) and treatment indicator (CEE versus placebo) as the exposure, while adjusting for metabolite levels at baseline, age, BMI, CHD case control status, race, prevalent diabetes and hypertension. The following acronyms are used to define metabolite classes: DAG or MAG: Di(Mono)acyl glycerol, TAG: triacylglycerol, GP: glycerophopholipid, LPC or LPC(P): Lysophosphatidyl choline or Lysophosphatidyl choline plasmalogen; LPE: Lysophosphatidyl ethanolamine; PC: Phosphatidyl choline; PC [P]: Phosphatidyl choline plasmalogen; PE: Phosphatidyl ethanolamine; PE[P]: Phosphatidyl ethanolamine plasmalogen; C&CE: Cholestyrl or Cholesteryl Ester; SL: Other sphingolipid; SM: sphingomyelin; FA: Fatty acid; AC: Acylcarnitine; AA: Amino acid; BA: Bile acid; PU&PY: purines and pyrimidines; QA: Quaternary amine; IND: Indole and indole derivatives; PYD: pyridines and derivatives; OA: Other organic acid; CARB: Carbohydrates and conjugates; BZ: Benzene and derivatives; OTH: other.

Figure 4: Metabolite classes enriched for metabolites showing concerted changes by active HT relative to placebo. NES denotes the normalized enrichment score from Metabolite set enrichment analysis (MSEA). A positive (negative) enrichment score corresponds to coordinated increases (decreases) in abundance of the metabolites in the active HT arm relative to placebo. * denotes metabolite classes with significant enrichment (FDR adjusted p value < 0.05).

The following acronyms are used to define metabolite classes: DAG or MAG: Di(Mono)acyl glycerol, TAG: triacylglycerol, GP: glycerophopholipid, LPC or LPC(P): Lysophosphatidyl choline or Lysophosphatidyl choline plasmalogen; LPE: Lysophosphatidyl ethanolamine; PC: Phosphatidyl choline; PC [P]: Phosphatidyl choline plasmalogen; PE: Phosphatidyl ethanolamine; PE[P]: Phosphatidyl ethanolamine plasmalogen; C&CE: Cholestyrl or Cholesteryl Ester; SL: Other sphingolipid; SM: sphingomyelin; FA: Fatty acid; AC: Acylcarnitine; AA: Amino acid; BA:

Bile acid; PU&PY: purines and pyrimidines; QA: Quaternary amine; IND: Indole and indole derivatives; PYR: pyridines and derivatives; OA: Other organic acid; CARB: Carbohydrates and conjugates; BZ: Benzene and derivatives; OTH: other.

Figure 5: Twelve metabolites with discordant HT effects in the Women's Health Initiative hormone trials (HT) and associated with CHD risk in the Women's Health Initiative Observational Study (WHI-OS).