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Pharmacogenomics of estrogens on changes in carotid artery intima-medial thickness and coronary arterial calcification – Kronos Early Estrogen Prevention Study

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Abstract

Background: Prior to the initiation of menopausal hormone treatment (MHT), genetic variations in the innate immunity pathway were found to be associated with carotid artery intima-medial thickness (CIMT) and coronary arterial calcification (CAC) in women (n=606) enrolled in the Kronos Early Estrogen Prevention Study (KEEPS). Whether MHT might affect these associations is unknown.

Methods: The association of treatment outcomes with variation in the same 764 candidate genes was evaluated in same KEEPS participants four years after randomization to either oral conjugated equine estrogens (0.45 mg/day), transdermal 17β estradiol (50 μg/day), each with progesterone (200 mg/day) for 12 days each month, or placebo pills and patch.

Results: Twenty SNPs within the innate immunity pathway most related with CIMT after 4 years were not among those associated with CIMT prior to MHT. In 403 women who completed the study in their assigned treatment group, SNPs within the innate immunity pathway were found to alter the treatment effect on 4-year change in CIMT (i.e. significant interaction between treatment and genetic variation in the innate immunity pathway; $p<0.001$). No SNPs by treatment effects were observed with changes of CAC >5 Agatston Units after 4 years.

Conclusion: Results of this study suggest that hormonal status may interact with genetic variants to influence cardiovascular phenotypes, specifically, the pharmacogenomic effects within the innate immunity pathway for CIMT.

Key words: atherosclerosis, candidate genes, estrogen, innate immunity, thrombosis

Introduction

Controversy surrounding whether or not menopausal hormone treatment (MHT) slows progression of cardiovascular disease reflects, in part, differences among studies in experimental design (timing of initiation of hormone treatment, doses and formulations) and co-existing cardiovascular risk factors of the population or study participants (4, 6, 16, 22, 24, 30, 33). In addition, potential benefit of MHT against development of atherosclerosis is offset by potential risk of venous thrombosis especially in women with genetic variation in factors associated with coagulation which may be more pronounced with the use of oral compared to transdermal estrogen products and the impact of the oral products on liver metabolism (8, 9, 12, 35). In addition, several studies have implicated genetic variants in estrogen receptors with progression of cardiovascular disease and venous thrombosis in men and women (1, 23, 34). However, estrogen receptor polymorphisms were not associated with adverse cardiovascular outcomes in women randomized to oral conjugated equine estrogens in the Women's Health Initiative (31). Therefore, much remains to be learned about phenotypic expression of polymorphisms in genes associated with estrogen responsiveness and thrombotic capacity in evaluating potential benefits and risk of MHT in postmenopausal women.

The Kronos Early Estrogen Prevention Study (KEEPS) was designed to evaluate the effects of MHT on progression of cardiovascular disease defined by quantitative changes in carotid artery intima-medial thickness (CIMT) and coronary artery calcification (CAC), accepted measures of subclinical atherosclerosis. A targeted candidate genetic analysis demonstrated no association of genetic polymorphisms in estrogen receptors α or β with the absolute value of either CIMT or CAC in women enrolled in KEEPS prior to randomization to treatment. Of the polymorphisms of genes within the anticoagulant, procoagulant, fibrinolytic or innate immunity pathways, only polymorphisms of genes within the innate and humoral immunity pathways were associated with the baseline absolute value of CIMT or CAC, respectively (26). However, at the

time of that study, women had transitioned into menopause and were estrogen depleted (serum 17β estradiol <40pg/mL). Thus, it remains to be determined whether estrogen treatments alter these associations. Therefore, associations of genetic variants within the same targeted candidate genes were evaluated with progression (i.e. change in measures) of subclinical atherosclerosis following randomization to either active estrogen treatments or placebo for four years. Two hypotheses were considered in these analyses: 1) genetic markers would be associated with change in CIMT or CAC after the 4 years independent of treatment, and, 2) there would be pharmacogenomic effects of genes with respect to change in CIMT or CAC after application of treatment or placebo (i.e. genetic markers will modify the effects of the treatment/placebo on CIMT and CAC progression.)

Methods

Participants: Women meeting inclusion criteria for the KEEPS (NCT00154180) and who gave informed consent to have their DNA used for research purposes were included in this study. There were nine centers participating in KEEPS: Brigham and Women's Hospital; Columbia University College of Physicians and Surgeons; the Kronos Longevity Research Institute; Mayo Clinic, Rochester, MN; Montefiore Medical Center; University of California at San Francisco; University of Utah, University of Washington; and Yale University. Each institutional review board of these participating institutions approved the study.

KEEPS inclusion/exclusion criteria are detailed elsewhere (15). Briefly, women were excluded from KEEPS if they had a history of, or were symptomatic for, cardiovascular disease; smoked more than ten cigarettes/day; had coronary artery calcification (i.e., ≥50 Agatston Units), body mass index >35 kg/m^2, dyslipidemia (low-density lipoprotein cholesterol >190 mg/dL), hypertriglyceridemia (triglycerides, >400 mg/dL), 17β-estradiol >40 pg/mL; uncontrolled hypertension (systolic blood pressure >150 mm Hg and/or diastolic blood pressure >95 mm Hg) or fasting blood glucose >126 mg/dL; or used lipid lowering drugs (15, 25). Women meeting inclusion criteria were randomized to treatment: oral conjugated equine estrogens (Premarin,

0.45mg/day), transdermal 17β-estradiol (via skin patch, Climara, 50μg/day) both with progesterone (oral Prometrium, 200 mg/day) for the first 12 days of the month, or placebo group (inactive pills/patch) for four years. Of women randomized to treatment, 684 consented to allow analysis of their DNA. Of these, 606 had clinical data, CIMT, and CAC measurements available for analysis prior to treatment (baseline). Follow-up data were available for 565 women at 1 year, 539 women at 2 years, 519 women at 3 years and 512 women at 4 years.

Clinical methodology and genotype quality control. All blood samples were collected after an overnight fast, frozen at -70^{o}C on site until they were either processed locally, or sent to the Kronos Science Laboratory (Phoenix, AZ, USA) for storage or assays. Genomic DNA was extracted from whole blood using the QIAamp DNA Blood Midi Kit (Qiagen), and the DNA concentration was measured by the PicoGreen technique (Invitrogen). The genotyping panel for identification of the SNPs for the custom 16,720 bead Illumina Infinium (13,229 SNPs including 492 ancestry informative markers (AIMs) (32) are described in detail elsewhere (17).

Clinical phenotypic characteristics, genotyping and quality control were performed as previously described (26). CIMT measured by B-mode ultrasound and CAC measured from non-enhanced cardiac computed tomography scans were quantified at Core reading centers for KEEPS (14, 15, 26). All CIMT scans were obtained with high-resolution ultrasonographic equipment using standardized methods for reproducing transducer angulation and cardiac gating (18, 19) by personnel at each site who were trained at the central reading center. The scans were read by trained personnel blinded to treatment assignment at the central reading center. The intima-media thickness of the far wall of the distal common carotid artery was determined as the average of 70 to 100 standardized measurements between the intima-lumen and media-adventitia interfaces by automated computerized edge detection software (patents obtained in 2005, 2006, and 2011). To determine variability of the readings, two scans were obtained at separate visits (from 3 days to 6 weeks apart) prior to randomization. As reported

previously, the mean coefficient of variation between these two readings was 0.6% (SD, 0.7 [range, 0.0% to 7.7%]) (14)

Statistical analysis: Clinical characteristics were summarized at baseline and 4 years separately, changes in clinical characteristics between baseline and the 4-year measurement were tested using Wilcoxon Signed Rank tests. The analysis consisted of two outcomes, 4-year change in CIMT as a continuous measure and 4-year change in CAC as a binary measure [change > 5 vs. ≤5 Agatston Units (AU)]. CIMT was measured at yearly intervals for 4 years, and modeling all 4-years of measurements yielded similar conclusions (data not shown). CAC was measured at baseline and at year 4. First, the relationships of the two outcomes (CIMT and CAC) and conventional cardiovascular risk variables were tested using linear regression and logistic regression, respectively. None of these variables were significantly correlated with the outcomes after multiple testing correction (data not shown), and were not adjusted for in subsequent genetic analyses. However, percentage of European ethnicity was adjusted for in subsequent genetic analyses to address possible population stratification. Previously, we had established using ancestry informative markers that most KEEPS participants were Caucasian and we used percentage CEU ancestry from the STRUCTURE program to estimate the proportion of European ancestry within each individual (26). STRUCTURE allows for population admixture and assigns individuals in the sample of interest (the KEEPS sample) population probabilities. The technique assumes the loci are unlinked and it assumes Hardy-Weinberg equilibrium within the populations.

Two genetic analyses were considered for each outcome, first testing for SNP effects on the outcomes, and secondly testing for SNP*treatment interactions (i.e., SNP affecting the relationship of the treatment and outcome, a pharmacogenomic effect). In both analyses, SNPs (as count of minor allele) were used to model change in CIMT and change in CAC, via linear and logistic regression, respectively. In the case of the pharmacogenomic analysis, a treatment main effect and treatment*SNP interaction were also modeled with the treatment*SNP

interaction being tested with a likelihood ratio test. To correct for multiple testing we estimated the effective number of independent tests and using the Bonferroni method we set our threshold of significance at $p<7.73E-06$ (11).

To test the overall association of SNPs in each of 4 genetic pathways (anticoagulant, procoagulant, fibrinolysis, and innate immunity; a complete list of SNPs was published previously (26)), a global test of the genetic variation in each pathway was conducted modeling 4-year change in CIMT or CAC by pathway SNPs using random effects models and testing all SNPs in a pathway simultaneously with a likelihood ratio test (13).

To the test the overall pharmacogenomic effect of pathways of SNPs on change in CAC and CIMT, the Principle Components (PC)-gamma method was used (3). Principal components were formed from SNPs within each gene, and enough principal components were retained for each gene to explain 80% of the variation in SNPs within that gene. Then gene level tests were conducted for each gene using F-tests of all treatment interactions with PCs retained in a regression model for the continuous change in CIMT; and likelihood ratio test for all treatment interactions with PCs retained in a logistic regression model of dichotomized change in CAC. Fisher's p-value combination method was then used to generate a test statistic for the pathway. Since genes in a pathway may be correlated to some degree, an asymptotic test was not used; instead, a parametric bootstrap approach was applied (7) to obtain a pathway level p-value. Briefly, we fit a null model without the PC*treatment interactions and for each individual calculated their fitted value. Next, a bootstrap sample of participants was taken with replacement, and a set of new CIMT or CAC values was simulated for the individuals in the sample. This process was repeated to generate 1000 bootstrap samples under the null hypothesis on no treatment-gene interactions. Each of these 1000 datasets was analyzed using the PC-gamma method to obtain an empirical distribution of test statistics for the gene-treatment interaction at the pathway level under the null hypothesis. The observed pathway test statistic was then compared to this empirical null distribution, with the p-value being the fraction of

empirical test-statistic values that were greater than or equal to the observed test-statistic. All analyses were performed in R v2.14.0.

Results

Clinical phenotypic characteristics of women for whom SNP analyses were performed over the four years of treatment are shown in Table 1.

CIMT

Independent of treatment: Mean CIMT increased over the four years of the study with variability increasing with time (Figure 1). To evaluate these increases two analyses were performed: one examining only the change in CIMT from baseline to 4 years and the second considering simultaneously all measures of CIMT over 4 years that included data of women with readings at multiple time points (longitudinal analysis). Results of these two analyses were similar, so for clarity, only changes from baseline to 4 years are presented here. None of the conventional cardiovascular risk factors (Table 1) measured at baseline or their change over the 4 years was found to be associated with changes in CIMT at 4 years (data not shown). The relationship between SNPs and the change in CIMT, adjusting for percentage European ancestry for an individual, did not identify any significant associations after correcting for the number of SNPs tested (Table 2, and Table 3). None of the SNPs previously reported to be associated with the absolute value of CIMT at baseline (i.e. prior to hormone treatment) (21) were here found to be associated with the change in CIMT after treatment. Furthermore, adjusting for baseline CIMT did not affect the outcomes.

Pharmacogenomic effect of SNPs on the relationship of treatment and changes in CIMT at 4 years. Of women with DNA for analysis, 403 completed the study in their assigned treatment group. Based on the P-value cutoff (see Methods), there were no statistically significant signals for any particular SNP with a pharmacogenomic effect (data not shown); however, SNPs in the innate immunity pathway had an overall pharmacogenomic effect on 4-year change in CIMT (interaction of SNPs in the genetic pathway and treatment) in these women ($P < 0.001$, Table

3). We also observed that the SNPs effects on the 4 year change in CIMT varied by treatments (i.e. presence of common or rare allele within transdermal 17β-estradiol or conjugated equine estrogens groups) compared to placebo (Table 4 and Figure 2).

CAC

Independent of treatment. Change in CAC between baseline and 4 years of treatment was dichotomized as change >5 AU (set by the sensitivity of the measurement) due to the large number of women with a CAC score of zero at baseline and after 4 years of treatment. Baseline clinical parameters showing significant association with the change in CAC were baseline fasting blood glucose, triglycerides and diastolic blood pressure (Table 5). However, only baseline CAC appeared to be associated with change in CAC > 5 AU ($P < 0.001$) after correcting for all of the generally accepted risk factors and was included as an adjustment factor in subsequent genetic analyses.

Using a stepwise algorithm of clinical variable effects in multivariable logistic models, the "best" predictive model for change in CAC included baseline CAC>0 AU ($P < 2e\text{-}16$), baseline triglycerides ($P = 0.009$), baseline weight ($p = 0.03$), and change in CIMT (4 yr-baseline; $P = 0.04$).

The relationship between SNPs and change in CAC was modeled first using multiple logistic regression, with adjustment for percentage European ancestry and baseline CAC > 0 (data not shown). None of the SNPs associated with changes of CAC > 5 AU after 4 years (Table 6) were among the SNPs of interest based on association with the absolute AU score for CAC prior to treatment (21).

Pharmacogenomic effect of SNPs on the relationship of treatment to changes in CAC at 4 years. In those women who completed the trial in their assigned treatment group or placebo over the 4 years (n=403), and after adjusting for ethnicity and CAC > 0 AU, there was little evidence for a SNP by treatment interaction effect (Table 7). Genes not within the innate immunity pathway that showed nominal significance for a pharmacogenomics effect of

treatment on CAC were SERAPINE 2 and beta adrenergic receptor 2 (ADRB2), respectively (Table 7).

Discussion

Changes in CIMT and CAC, the main outcomes of KEEPS, were not significantly altered by either oCEE or tE2 compared to placebo over the 4 years of the study (14). In the present study, a targeted candidate gene analysis provides insight into processes contributing to these main outcomes by identifying significant gene-by-treatment interactions in the progression of CIMT but not CAC.

CIMT increases with age and time past menopause (38). The absence of association of increases in CIMT with conventional risk factors most likely reflects the narrow range for most of these variables (Table 1) and, in particular, mean systolic blood pressure did not differ significantly from baseline to year 4 and was within what would be considered clinically a "normative" range. Variability in progression of CIMT reflects in part natural aging processes reflected by chronological and menopausal age, treatment assignment and gene/treatment interactions.

Genes of the innate immunity pathway associated with the absolute value of CIMT at baseline prior to randomization to treatment (26). Using pathway analysis, the innate immunity pathway associated with pharmacogenomic effects of the hormone treatments on changes in CIMT. Although individual SNPs did not achieve statistical significance with the change in CIMT, the SNPs altered the treatment effect (Figure 2). Depending upon the mean allele frequency in the population, the penetrance of this pharmacogenomic effect would manifest as increased variance in the phenotype (Figure 1) and reduce the ability to differentiate among groups, a result consistent with the cumulative analysis of the KEEPS cohort (14).

Other studies have identified associations of genes related to the immunity pathway with increases in CIMT (37). However, some genome-wide association studies fail to identify genes

associated with increases in CIMT, which may reflect that these studies do not perform hormone stratified analysis nor account for sex as a dichotomous variable in the analysis. (28). Apolipoprotein E 4 (*ApoE4*) was shown to be associated with CIMT in several studies (21) but unfortunately, this gene/SNP was not tested in the present study. Examining *ApoE4* might be interesting as this gene/SNP has greater critical risk factor for Alzheimer's disease in women compared to men (29) and a sex differential of this gene/SNP in association with other phenotypes would be interesting to explore.

Although fasting blood glucose, triglycerides and diastolic blood pressure associated with CAC in univariate analysis, these conventional cardiovascular risk factors did not remain significant after correction for multiple testing, which may reflect that the KEEPS participants were relatively healthy women with conventional cardiovascular risk factors within normative ranges. The first two variables relate to energy utilization while diastolic blood pressure may reflect general arterial stiffening/reduced compliance in post-menopausal women (10, 27, 36) and may become more relevant with aging.

One gene not within the innate immunity pathway that showed nominal association with CAC, SERPINE2, may impact the coagulation cascade as this gene encodes a protein that inhibits thrombin and urokinase plasminogen activator type 2. In addition, the adrenergic system may impact CAC as changes in beta adrenergic receptors are associated with changes in total peripheral resistance in women after menopause and variants in the receptor are implicated in response to beta adrenergic blockers used in the treatment of hypertension (2, 20). Conventional risk factors such as hypertension, hypercholesterolemia and Type 2 Diabetes (T2D) exacerbate accumulation of calcium in the coronary arteries. In expression of complex traits, and despite the weak genetic effects, certain genetic variants in metabolic or immune pathways may become more important mechanisms of disease progression. For example, persons with T2D, CAC negatively correlated with variants in CD40 (5). Also, in women

screened for KEEPS, 14% were excluded based on CAC without other risk factors (25), suggesting that in women, at least, coronary calcification may have several etiologies.

This study has several limitations. The study included a small number of mostly Caucasian women who were at low risk for cardiovascular disease. It would be interesting to observe these women over a longer time-course, even in the absence of hormone treatment, to determine whether the same or different SNPs associate with disease progression with aging. Serum levels of hormones were not consistently above that defining menopause for women randomized to treatment and different associations might be found if serum levels of estrogen were increased further. As in the previous analysis, polymorphisms in estrogen receptors did not associate with either phenotype. Estrogen response elements reside in the promoter regions of many genes and affect gene expression, which was not measured in the present study. Influences of MHT on gene expression would be expected to differ among formulations given that the metabolites of estrogen have differing binding affinity for estrogen receptors. In the present study, the differential expression of genes with the SNPs of interest in response to estrogen determined the contribution of those including perhaps those regulated by estrogen and the phenotype. Additional work is needed to understand how polymorphisms in estrogen receptors contribute to development of cardiovascular disease in women.

The magnitude of the pharmacogenomics effects differed by type of MHT. It is unlikely that methodological variability in measurement of CIMT contributed to the pharmacogenomic effects as methods to obtain the ultrasound scans were standardized, performed by trained personnel and coefficient of variations between two scans obtained prior to randomization was <1%. Power to detect pharmacogenomic effects on change in CAC was limited by the small number of women with changes of CAC >5 Agatston Units, as the approach that was used to assess significance of treatment-by-gene interactions at the pathway level (PC-Gamma method with the parametric bootstrap) is expected to have low power for evaluating association with dichotomous outcomes when the number of cases is small. Finally, the study used a candidate

gene approach specifically to investigate genes related to coagulation and immunity to evaluate the genetic components of MHT and thrombotic risk. Other pathways may have been discovered with a genome wide association approach.

In spite of these limitations, the results of this study emphasize that in genetic association studies of complex disease traits, it is critical to account for sex and hormonal status of the study participants. In addition, this analysis provides value to the scientific community as it is the first study to identify pharmacogenomic effects of MHT on vascular remodeling and thus, provides insight into why there is variability in cardiovascular effects of MHT in women.

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Virginia M. Miller conceived the idea for the study, participated in oversight of data collection, analysis and preparation of the manuscript.

Gregory D. Jenkins performed the statistical analysis, interpretation and preparation of manuscript.

Joanna M. Biernacka assisted with the component pathway analysis.

John A. Heit participated in conceptual aspects of the genotyping study including design of the custom gene array, interpretation of the data and intellectual content, preparation and editing of the manuscript.

Gordon S. Huggins oversaw preparation of the DNA plates for the KEEPS trial and contributed to editing the manuscript.

Howard N. Hodis oversaw measurements of carotid artery intima-medial thickness and provided intellectual content, editing and proof reading of the manuscript.

Matthew J Budoff participated in oversight of data collection and measurements of coronary artery calcification, and assisted in preparation of the manuscript.

Rogerio A. Lobo contributed to the original design of KEEPS, intellectual content and editing of the manuscript.

Hugh S. Taylor contributed to intellectual content and editing of the manuscript.

JoAnn E. Manson contributed to the original design of KEEPS, intellectual content and editing of the manuscript.

Dennis M. Black oversaw the statistical analysis for the KEEPS trial.

Frederick Naftolin, co-principal investigator for the KEEPS trial, participated in the general design of the KEEPS and provided editing of the manuscript.

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567

568 **Figure Legends**
569

570 Figure 1. Median, standard deviation and 95% confidence intervals of changes in carotid artery
571 intima-medial thickness from baseline (prior to randomization, n = 606), at 1 year (n = 565), 2
572 years (n = 539), 3 years (n = 519) and 4 years (n = 512) after randomization but independent of
573 treatment assignment in women enrolled in KEEPS.
574

575 Figure 2. Depiction of SNPs on three genes with the lowest p-values of pharmacogenomic
576 association (interaction of SNP and treatment).with respect to change in carotid artery intima-
577 medial thickness (CIMT) from baseline to 4 year in women who completed the study in each
578 treatment randomized treatment assignment or placebo (n = 160) transdermal17β estradiol (E2,
579 n = 119) or oral conjugated equine estrogen (CEE, n = 123). Data are shown as median, the
580 box is the 25th and 75th percentile range; vertical lines represent the 1.5 interquartile range
581 (IQR); points outside the IQR are plotted as is (outliers). Upper panel: r11466536 for gene
582 TGFBR2, transforming growth factor beta receptor 2 binding transforming growth factor beta,
583 the complex phosphorylates proteins and acts as a transcription factor regulating cell
584 proliferation; Middle panel: r1569723 for gene CD40, a protein of the tumor necrotic factor
585 superfamily of receptors involved in triggering immunological activation; Bottom panel: r261060
586 for gene DOCK2, dictator of cytokinesis 2, encodes a protein involved with small G-protein
587 coupled intracellular signaling. Although these individual SNPs within the innate immunity
588 pathway did not reach statistical significance, the overall pathway analysis which represents the
589 collective analysis of all of the genes/SNPS included in this study did reach statistical
590 significance.

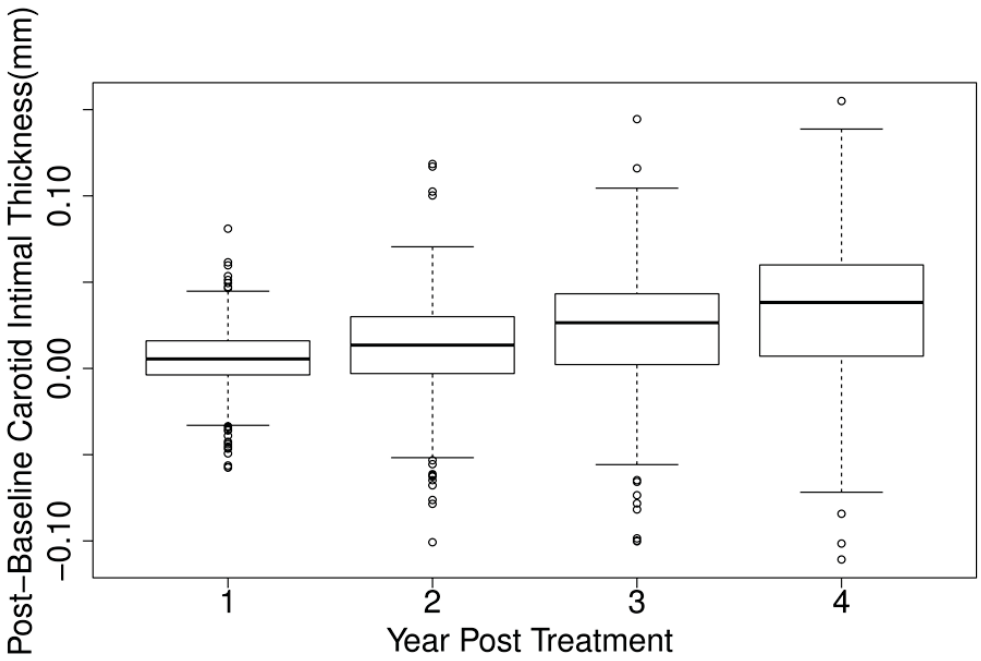
Post−Baseline Carotid Intimal Thickness(mm)
−0.10
0.00
0.10
1
2
3
4
Year Post Treatment

Figure 1

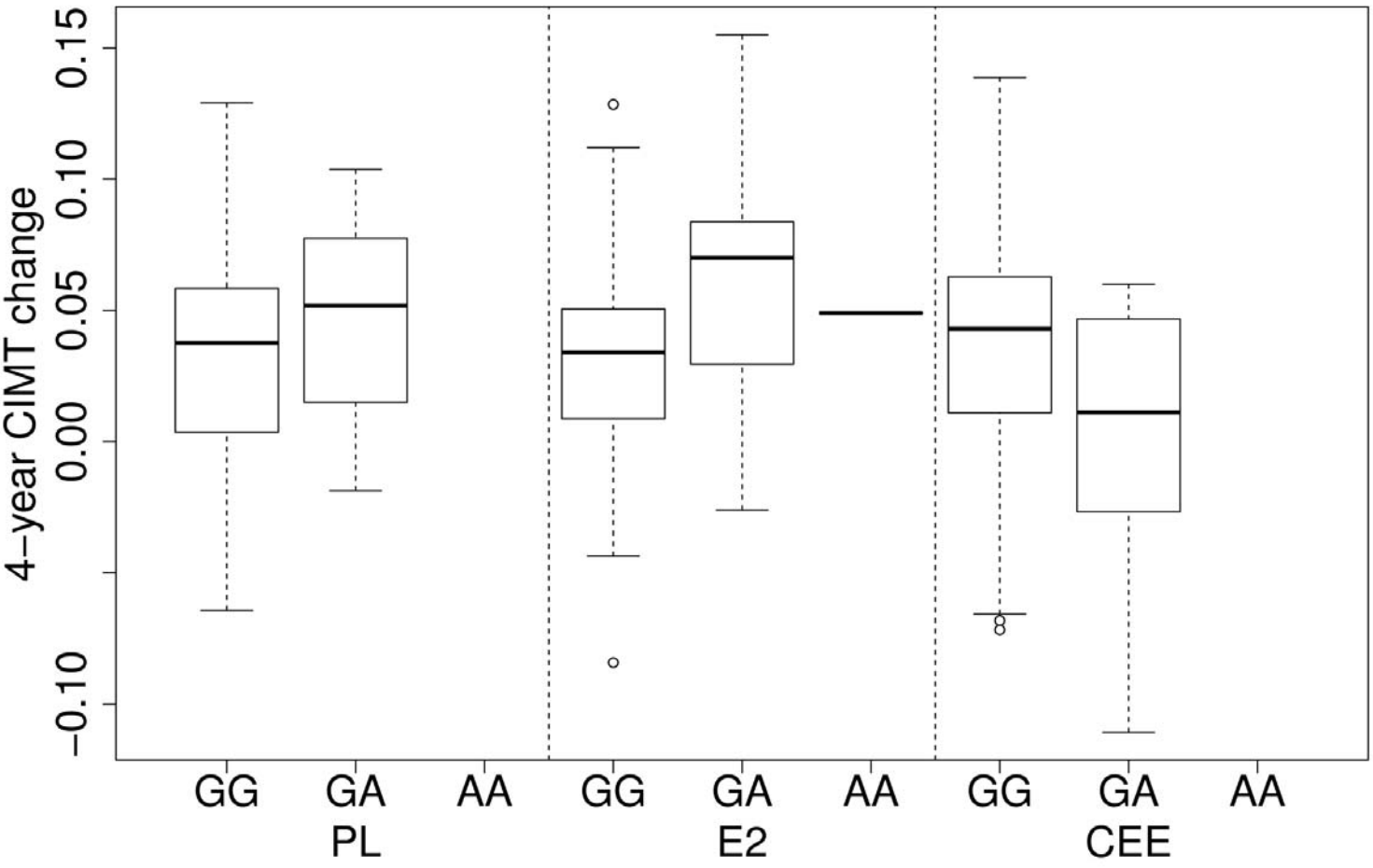

Figure 2 – Top panel

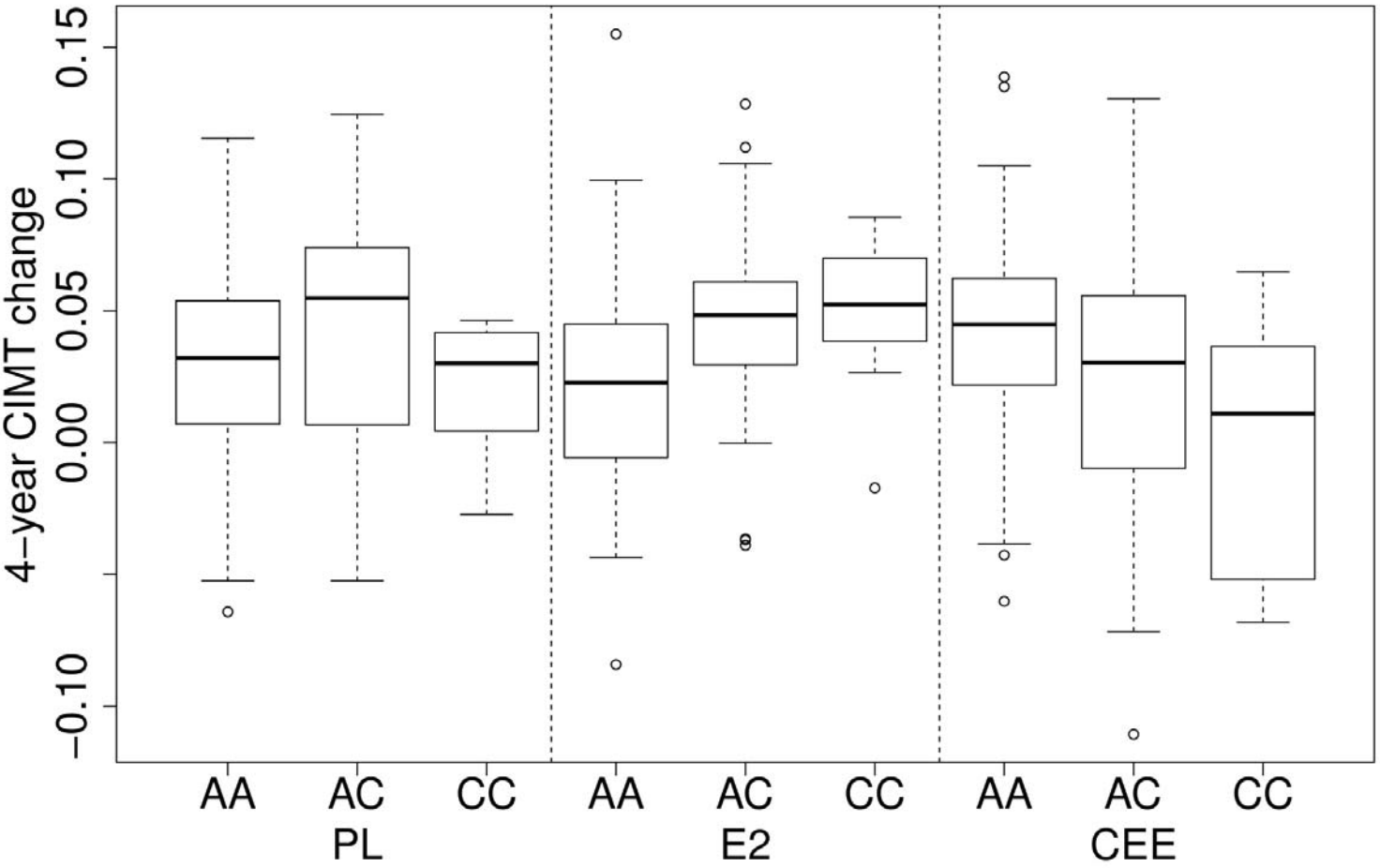

Figure 2 – middle panel

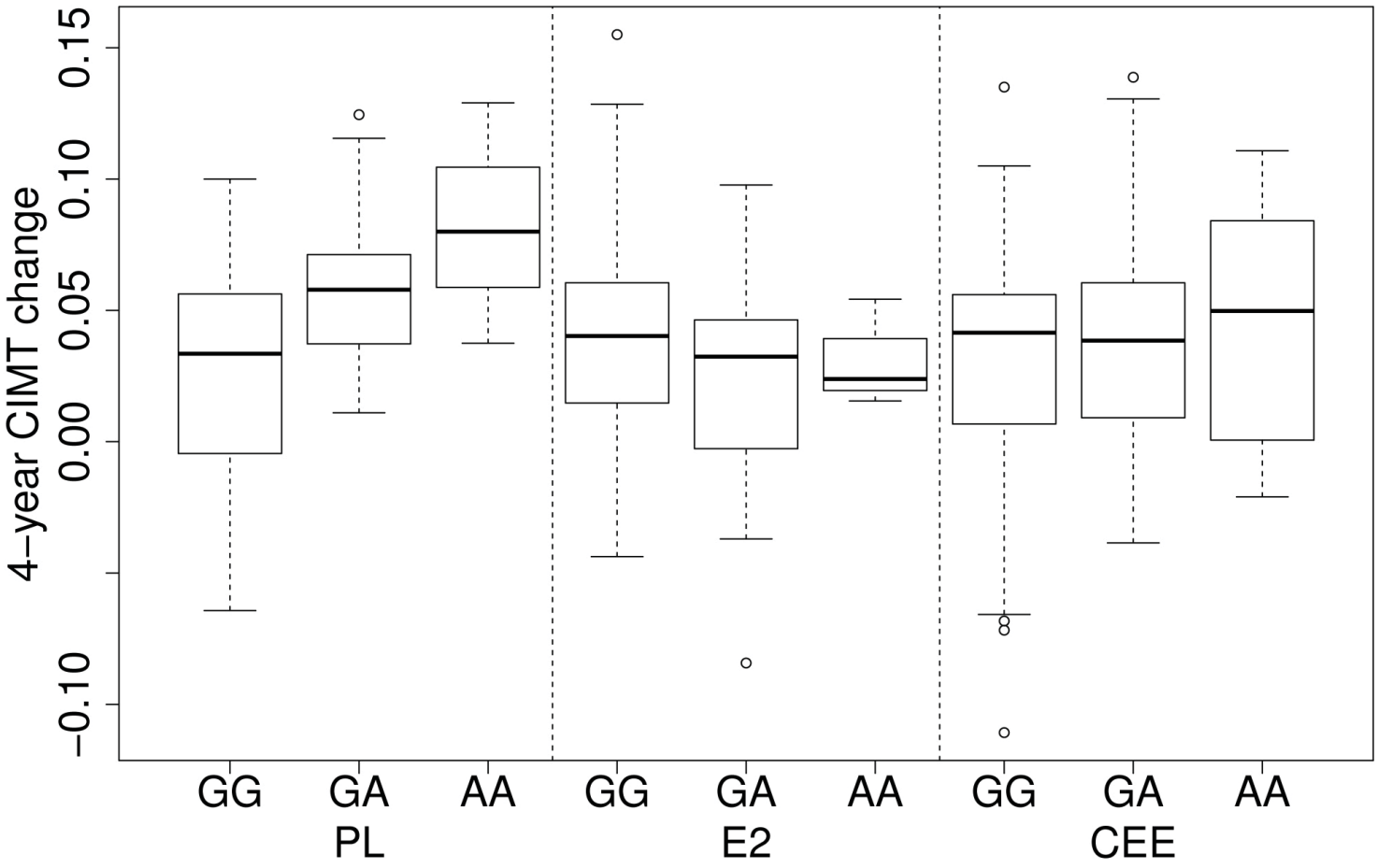

Figure 2 – bottom panel

Table 1. Phenotypic characteristics of KEEPS participants in the genetic association analysis.

	Baseline (pre-treatment)	4-years post treatment	P-value
Treatment, no. participants			
A = premarin	188	157	-
B = patch	186	161	-
C = placebo	232	194	-
Weight (kg)[a]	70.6(11.8)	71.1(12.5)	<0.01
Body mass index (kg/m$^{2)}$	26.3(4.3)	26.6(4.62)	<0.01
Waist circumference (cm)	84.9(11.7)	85.1(11.5)	0.20
Systolic blood pressure (mm Hg)	119(15.1)	118(13.9)	0.95
Diastolic blood pressure (mm Hg)	75.1(9.26)	73.7(9.1)	0.02
C-reactive protein (pg/mL)	2.18(3.38)	3.05(4.38)	<0.01
Fasting blood glucose (mg/dL)	79.7(9.57)	81.3(9.28)	<0.01
Total cholesterol (mg/dLl)	208(34.5)	210(35.7)	0.11
High density lipoprotein cholesterol (mg/dL)	72.1(14.6)	73.1(15.1)	0.08
Low density lipoprotein cholesterol (mg/dL)	111(28.4)	111(30.7)	0.65
Triglycerides (mg/dL)	86.3(54.9)	90.8(52.3)	<0.01
Insulin (pmol/L)	6.01(7.65)	5.23(6.38)	0.01
HOMA-IR score	1.23(1.74)	1.07(1.28)	0.09

[a]Data are shown as mean (Standard Deviation); P values depict difference between baseline and year 4 independent of treatment by Wilcoxon signed ranks test. HOMA-IR = Homeostasis Model Assessment of Insulin Resistance

Table 2. 20 SNPs with the smallest P-values of longitudinal association with changes in carotid intima medial thickness (CIMT) at 4 years of treatment in women enrolled in KEEPS.

SNP	gene	chromosome	position (bp)	common allele	rare allele	MAF	SNP call rate	Estimated Effect on change in CIMT (difference in 1 minor allele)	SE of estimate	p-value
rs6884061	TNFAIP8	5	118711330	G	A	0.204	1.000	1.184E-02	3.177E-03	2.164E-04
rs12848910	CYBB	23	37551189	A	G	0.065	0.990	-1.992E-02	5.432E-03	2.711E-04
rs4896243	LOC100131120//IFNGR1	6	137556483	A	G	0.447	1.000	-9.243E-03	2.545E-03	3.098E-04
rs1860545	TNFRSF1A//SCNN1A//PLEKHG6	12	6317038	G	A	0.352	0.979	-1.003E-02	2.834E-03	4.402E-04
rs4850994	IL1R2	2	102020660	G	A	0.139	0.995	-1.234E-02	3.578E-03	6.088E-04
rs11954573	F2R	5	76070823	G	A	0.288	0.985	9.789E-03	2.894E-03	7.743E-04
rs17027013	IMMT	2	86263580	T	A	0.458	0.998	-8.620E-03	2.587E-03	9.270E-04
rs2341746	COLEC12	18	495472	A	C	0.232	0.998	1.019E-02	3.063E-03	9.465E-04
rs17037397	MTHFR//CLCN6	1	11784750	C	A	0.045	0.998	1.985E-02	5.969E-03	9.474E-04
rs2274976	MTHFR//C1orf167	1	11773514	G	A	0.046	0.995	1.964E-02	5.915E-03	9.623E-04
rs1027702	DUSP12	1	159979481	G	A	0.409	1.000	9.138E-03	2.768E-03	1.031E-03
rs264846	DOCK2	5	169059316	A	T	0.369	0.998	9.133E-03	2.768E-03	1.039E-03
rs6707029	IMMT	2	86253595	A	G	0.459	0.990	-8.448E-03	2.587E-03	1.167E-03
rs1801131	MTHFR//C1orf167	1	11777063	A	C	0.296	0.998	9.029E-03	2.781E-03	1.245E-03
rs12649582	ANXA5	4	122832341	A	G	0.482	0.998	8.473E-03	2.618E-03	1.290E-03
rs2296135	IL15RA	10	6034700	C	A	0.481	1.000	8.092E-03	2.516E-03	1.385E-03
rs2153875	ITGB1	10	33230573	A	C	0.289	1.000	-9.190E-03	2.866E-03	1.429E-03
rs4951771	KIAA1522//YARS	1	33005810	A	G	0.311	0.998	-8.893E-03	2.813E-03	1.667E-03
rs1360151	C8A	1	57136629	G	A	0.141	1.000	1.154E-02	3.683E-03	1.831E-03
rs2871444	IL1R2	2	101979282	A	G	0.315	0.997	-9.049E-03	2.888E-03	1.832E-03

Table 3: P-values from Pathway analysis of SNPs in 4-pathways for association with CIMT or CAC for direct genetic or pharmacogenetic effects.

Phenotype	Type of effect	Anticoagulant	Fibrinolysis	Innate Immunity	Procoagulant
CIMT	Genetic only	0.381	0.849	0.316	0.051
CIMT	Pharmacogenetic	0.299	0.220	< 0.001	0.062
CAC>5	Genetic only	0.015	0.808	0.251	0.516
CAC>5	Pharmacogenetic	0.446	0.835	0.303	0.941

Table 4. 20 SNPs in the Innate Immune Pathway with the smallest p-values of pharmacogenomic association (interaction of SNP and treatment with changes in carotid artery intima-medial thickness (CIMT) at 4 years of treatment in women enrolled in KEEPS who completed the study in their assigned treatment group.

							Estimate (SE) for treatment vs placebo (given genotype) adjusting for ethnicity						
SNP	CHR	position(bp)	wild/minor allele	MAF	SNP call rate	Gene	E2/CC	E2/CR	E2/RR	CCE/CC	CCE/CR	CCE/RR	p.value
rs11466536	3	30710160	G/A	0.060	1.000	TGFBR2	-0.002(0.005)	0.012(0.013)	0.026(0.026)	0.006(0.005)	-0.042(0.014)	-0.090(0.029)	1.59E-04
rs1569723	20	44175471	A/C	0.249	0.970	CD40	-0.005(0.006)	0.006(0.006)	0.016(0.013)	0.012(0.006)	-0.013(0.007)	-0.039(0.014)	1.81E-04
rs261060	5	169258640	G/A	0.143	1.000	DOCK2*	0.0104(0.006)	-0.031(0.009)	-0.072(0.018)	0.004(0.006)	-0.019(0.008)	-0.042(0.017)	2.26E-04
rs776514	3	10250475	G/A	0.431	1.000	IRAK2	0.019(0.008)	-0.002(0.005)	-0.022(0.009)	0.024(0.008)	-0.002(0.005)	-0.027(0.009)	2.47E-04
rs7768807	6	353246	A/G	0.267	0.998	IRF4	0.0103(0.007)	-0.008(0.006)	-0.025(0.012)	-0.009(0.007)	0.009(0.006)	0.028(0.013)	2.66E-04
rs4073829	16	80527689	G/C	0.359	0.997	PLCG2	0.007(0.007)	-0.001(0.005)	-0.008(0.011)	0.022(0.007)	-0.008(0.005)	-0.037(0.011)	3.11E-04
rs138981	22	41927759	G/A	0.151	1.000	SCUBE1	-0.003(0.006)	0.008(0.007)	0.018(0.015)	-0.011(0.006)	0.030(0.009)	0.071(0.018)	3.80E-04
rs4791035	17	62237690	G/C	0.457	1.000	PRKCA	-0.0003(0.008)	0.001(0.005)	0.002(0.009)	0.023(0.008)	-0.001(0.005)	-0.026(0.009)	4.19E-04
rs261054	5	169261062	G/A	0.137	0.995	DOCK2*	0.009(0.006)	-0.031(0.009)	-0.071(0.019)	0.004(0.006)	-0.019(0.009)	-0.043(0.017)	4.44E-04
rs9378805	6	362727	A/C	0.443	1.000	IRF4	-0.003(0.008)	0.0002(0.006)	0.003(0.009)	0.021(0.008)	-0.002(0.005)	-0.026(0.009)	5.49E-04
rs261072	5	169248202	A/G	0.089	1.000	DOCK2*	0.009(0.005)	-0.038(0.011)	-0.085(0.023)	0.003(0.005)	-0.017(0.011)	-0.035(0.022)	6.12E-04
rs8056564	16	80537520	A/G	0.489	1.000	PLCG2	0.007(0.008)	0.0005(0.005)	-0.006(0.008)	0.025(0.008)	-0.0002(0.005)	-0.025(0.008)	7.88E-04
rs2243191	1	205082580	G/A	0.239	1.000	IL19	0.0122(0.006)	-0.012(0.007)	-0.037(0.013)	0.013(0.006)	-0.015(0.007)	-0.042(0.014)	7.91E-04
rs3774934	4	103646506	G/A	0.089	1.000	NFKB1	0.004(0.005)	-0.014(0.011)	-0.032(0.022)	0.007(0.005)	-0.042(0.012)	-0.091(0.024)	8.05E-04
rs12598402	16	80526349	A/G	0.442	1.000	PLCG2	0.003(0.008)	0.001(0.005)	-0.002(0.009)	0.022(0.008)	-0.002(0.005)	-0.027(0.009)	8.18E-04
rs8056122	16	31335179	A/G	0.413	1.000	ITGAD	0.006(0.008)	-0.001(0.005)	-0.008(0.009)	-0.015(0.007)	0.004(0.005)	0.026(0.010)	8.42E-04
rs261071	5	169249624	G/A	0.130	1.000	DOCK2*	0.010(0.006)	-0.029(0.009)	-0.069(0.019)	0.003(0.006)	-0.013(0.009)	-0.029(0.017)	9.14E-04
rs7736549	5	79415294	C/A	0.149	0.998	THBS4**	-0.005(0.006)	0.016(0.008)	0.036(0.017)	0.005(0.006)	-0.014(0.008)	-0.032(0.017)	9.16E-04
rs518162	11	100505711	G/A	0.104	1.000	PGR	0.009(0.005)	-0.033(0.011)	-0.074(0.023)	0.001(0.005)	0.001(0.0104	0.0011(0.021)	9.36E-04
rs264827	5	169054785	A/G	0.332	0.979	DOCK2	0.019(0.007)	-0.009(0.006)	-0.037(0.011)	0.006(0.007)	-0.003(0.005)	-0.013(0.011)	9.63E-04

*next to LOC100131897

**next to LOC100129870

Abbreviations: E2, transdermal 17β estradiol; CEE, oral conjugated equine estrogens; MAF, mean allele frequency

Table 5. Clinical variables prior to randomization (baseline) and change in CAC > 5 AU* after 4 years of randomization in women of KEEPS.

Clinical parameter at baseline	N	Odds Ratio	p-value
Age	495	1.016	0.76
Months past menopause	495	1.063	0.283
Body mass index	495	1.011	0.74
Systolic blood pressure	495	1.015	0.11
Diastolic blood pressure	495	1.038	0.01
Pulse pressure	495	1.001	0.91
Fasting blood glucose	495	1.029	0.03
Insulin	495	1.006	0.71
Total cholesterol	495	1.002	0.54
High density lipoprotein cholesterol	495	0.990	0.29
Low density lipoprotein cholesterol	495	1.004	0.36
Triglycerides	495	1.005	0.02
Interleukin-6	495	0.982	0.29
High sensitivity C-reactive protein	494	0.927	0.20
European Ancestry	495	0.681	0.45
Baseline CAC	495	1.471	<0.001

* AU = Agatston Units

Table 6. 20 SNPs with the smallest P-values of association with change in CAC > 5 AU* after 4 years of treatment in women enrolled in KEEPS.

SNP	Gene	chromosome	position(bp)	common allele	minor allele	mean allele frequency	SNP call rate	OR CAC>5 for 1 Minor allele difference	SE log OR	p-value
rs762484	F3	1	94776998	A	G	0.242	0.998	3.169	0.281	4.05E-05
rs7761846	ESR1	6	152254201	A	G	0.120	1.000	3.612	0.353	2.73E-04
rs854541	PPP1R9A//PON1	7	94758416	G	A	0.443	1.000	0.379	0.273	3.78E-04
rs3753019	COL18A1//SLC19A1	21	45749213	G	A	0.295	1.000	2.478	0.264	5.93E-04
rs11159198	ESRRB	14	75937134	G	A	0.411	1.000	0.352	0.304	6.03E-04
rs7115100	CADM1	11	114673869	A	C	0.114	0.995	2.732	0.296	6.77E-04
rs7944529	CADM1	11	114657017	A	T	0.123	0.998	2.746	0.298	7.11E-04
rs17686640	PRKCA	17	62048816	G	A	0.061	1.000	4.105	0.422	8.10E-04
rs9623806	SCUBE1	22	42015152	G	A	0.141	0.997	0.187	0.503	8.60E-04
rs2854946	SERPINA5	14	94118132	G	C	0.230	1.000	0.325	0.342	1.00E-03
rs3814415	EDNRA	4	148632039	A	G	0.160	0.998	2.918	0.326	1.01E-03
rs2017424	TNFRSF21	6	47376942	G	C	0.470	0.997	2.439	0.272	1.05E-03
rs2072474	IL1R2	2	102005641	A	G	0.200	1.000	2.553	0.290	1.21E-03
rs3759333	LTBR//SCNN1A	12	6362208	G	A	0.257	0.998	2.340	0.263	1.22E-03
rs6055955	PLCB1	20	8552181	A	G	0.479	0.997	2.364	0.266	1.23E-03
rs11567699	IL7R	5	35894768	G	C	0.284	1.000	2.387	0.271	1.33E-03
rs11719243	IL1RAP	3	191719795	A	G	0.254	1.000	2.338	0.269	1.60E-03
rs3194051	IL7R	5	35912031	A	G	0.283	0.998	2.357	0.272	1.65E-03
rs4876435	COLEC10	8	120156387	G	A	0.231	1.000	2.387	0.281	1.93E-03
rs1885550	SFTPD	10	81702420	G	A	0.187	1.000	2.551	0.302	1.95E-03

* AU=Agatston Units

Table 7. 20 SNPs with smallest P-values of pharmacogenetic association (interaction of SNP and treatment) with change in coronary artery calcification (change in CAC > 5 AU*) after 4 years of treatment in women enrolled in KEEPS who completed the study in their treatment.

							OR(95% CI) for treatment vs. placebo (given genotype) adjusting for ethnicity and baseline CAC>0 AU						
SNP	gene	CHR	position(bp)	wild/minor allele	MAF	SNP call rate	E2/CC	E2/CR	E2/RR	CCE/CC	CCE/CR	CCE/RR	p-value
rs3802857	CADM1	11	114583828	C/G	0.357	1.000	0.105(0.021,0.513)	2.01(0.703,5.77)	38.7(4.73,317)	0.19(0.038,0.947)	1.18(0.383,3.61)	7.27(0.683,77.3)	2.42E-04
rs2250889	MMP9*	20	44075813	G/C	0.080	0.998	0.461(0.156,1.36)	NA	NA	0.573(0.208,1.58)	NA	NA	2.87E-04
rs669607	C3orf68	3	28046448	A/C	0.439	1.000	3.21(0.768,13.4)	0.757(0.283,2.03)	0.178(0.026,1.24)	0.026(0.002,0.447)	0.272(0.072,1.04)	2.84(0.59,13.7)	3.54E-04
rs10738763	TEK	9	27105768	A/G	0.226	1.000	0.391(0.123,1.25)	6.44(1.15,36.2)	106(2.99,3770)	0.131(0.029,0.596)	3.82(0.702,20.8)	111(3.67,3370)	3.57E-04
rs615375	TEK	9	27102311	A/C	0.269	0.998	0.337(0.099,1.14)	7.86(1.31,47.3)	184(4.26,7910)	0.129(0.027,0.622)	4.56(0.773,26.9)	161(4.27,6040)	4.16E-04
rs8083599	COLEC12	18	362837	C/A	0.291	1.000	3.84(1.01,14.6)	0.456(0.142,1.46)	0.054(0.005,0.547)	3.94(0.96,16.2)	0.224(0.062,0.819)	0.013(0.001,0.195)	4.22E-04
rs343321	PLSCR1	3	147716959	G/A	0.125	1.000	0.802(0.287,2.24)	6.07(0.527,70)	46(0.327,6470)	0.191(0.052,0.708)	19.7(1.71,226)	2020(12.7,321000)	6.36E-04
rs11583394	IL19	1	205035516	A/G	0.225	1.000	1.02(0.317,3.25)	1.16(0.309,4.35)	1.32(0.0844,20.7)	1.88(0.593,5.93)	NA	NA	6.75E-04
rs9276976	HLA-DOA	6	33081772	G/A	0.148	1.000	0.274(0.08,0.944)	10.7(1.97,58.2)	417(11.4,15200)	0.413(0.128,1.34)	2.57(0.36,18.4)	16(0.275,927)	6.83E-04
rs1983357	LOC730057	3	64969335	A/C	0.223	1.000	2.24(0.66,7.62)	0.39(0.099,1.53)	0.068(0.004,1.15)	2.6(0.755,8.92)	0.043(0.004,0.422)	7.0e-4(6.0e-6,0.082)	7.84E-04
rs2292483	TRAF5	1	209599650	A/G	0.265	0.998	0.243(0.068,0.87)	2.95(0.873,9.96)	35.8(3.07,418)	0.621(0.189,2.04)	0.702(0.151,3.25)	0.792(0.035,18)	8.31E-04
rs9323910	SERPINA3**	14	94158379	G/C	0.247	0.998	4.75(1.35,16.8)	0.268(0.078,0.917)	0.015(0.001,0.206)	1.61(0.394,6.57)	0.272(0.068,1.08)	0.046(0.002,0.933)	8.61E-04
rs4252287	IL10RA	11	117373848	G/A	0.095	1.000	1.83(0.672,4.97)	NA	NA	1.09(0.377,3.17)	NA	NA	8.63E-04
rs12654778	ADRB2	5	148185934	G/A	0.374	1.000	0.257(0.051,1.29)	1.15(0.427,3.09)	5.12(0.84,31.4)	1.5(0.404,5.58)	0.307(0.077,1.23)	0.063(0.004,1.13)	9.19E-04
rs3794660	IRF8	16	84500669	C/G	0.034	1.000	0.972(0.387,2.45)	1.42(0,Inf)	NA	0.35(0.112,1.09)	NA	NA	1.03E-03
rs10406069	CD22+//	19	40528370	G/A	0.157	1.000	0.507(0.177,1.45)	NA	NA	0.344(0.104,1.14)	NA	NA	1.09E-03
rs10191694	SERPINE2++	2	224565510	C/A	0.380	1.000	2.08(0.544,7.92)	0.805(0.299,2.17)	0.312(0.046,2.11)	3.52(0.91,13.6)	0.083(0.009,0.736)	0.002(2.2e-05,0.174)	1.13E-03
rs1042713	ADRB2	5	148186633	G/A	0.399	1.000	0.238(0.048,1.19)	1.13(0.422,3.03)	5.36(0.93,30.8)	1.3(0.351,4.78)	0.312(0.078,1.25)	0.075(0.004,1.33)	1.15E-03
rs13068939	ITPR1	3	4563300	G/A	0.257	1.000	1.42(0.42,4.79)	0.716(0.199,2.58)	0.361(0.024,5.51)	0.115(0.022,0.601)	1.44(0.467,4.42)	18(1.85,175)	1.15E-03
rs894685	C1QL1	17	40410565	A/G	0.301	1.000	0.369(0.101,1.34)	1.8(0.578,5.61)	8.8(0.992,78)	1.35(0.424,4.28)	0.288(0.052,1.61)	0.061(0.002,2.03)	1.18E-03

*next to LOC100128028; **next to LOC390503; + next to FFAR1; ++ next to LOC100129171

NA = no results due to small sample size.

Abbreviations: E2, transdermal 17β estradiol; CEE, oral conjugated equine estrogen; MAF, mean allele frequency.

* AU=Agatston Units