Genetic polymorphisms associated with carotid artery intima-media thickness and coronary artery calcification in women of the Kronos Early Estrogen Prevention Study

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Miller VM, Pettersson TM, Jeavons EN, Lnu AS, Rider DN, Heit JA, Cunningham JM, Huggins GS, Hodis HN, Budoff MJ, Santoro N, Hopkins PN, Lobo RA, Manson JE, Naftolin F, Taylor HS, Harman SM, de Andrade M. Genetic polymorphisms associated with carotid artery intima-media thickness and coronary artery calcification in women of the Kronos Early Estrogen Prevention Study. Physiol Genomics 45: 79–88, 2013. First published November 27, 2012; doi:10.1152/physiolgenomics.00114.2012.—Menopausal hormone treatment (MHT) may limit progression of cardiovascular disease (CVD) but poses a thrombosis risk. To test targeted candidate gene variation for association with subclinical CVD defined by carotid artery intima-media thickness (CIMT) and coronary artery calcification (CAC), 610 women participating in the Kronos Early Estrogen Prevention Study (KEEPS), a clinical trial of MHT to prevent progression of CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, or innate immunity pathways. According to linear regression, proportion (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, CVD, were genotyped for 13,229 single nucleotide polymorphisms (SNPs) with...
coagulation cascade potentially provide a link between development of subclinical cardiovascular disease and thrombosis. Variation in plasma concentrations of procoagulant factors and activation markers of coagulation and fibrinolysis exhibit a high degree of heritability among apparently healthy individuals (4, 18, 59, 71, 83, 84). Mutations may occur in several pathways: 1) components of the anticoagulant pathway, 2) mutations that down-or upregulate the procoagulant pathway, 3) mutations that downregulate fibrinolysis, and 4) mutations of the immunological pathway (69). However, isolated impairment of one of these pathways is insufficient to cause thrombosis (10, 28, 59). Thus, in the setting of changes in hormonal status (estrogen-deplete or -replete), interactions among pathways linking hemostasis to cardiovascular risk represent complex genetic phenomena. Indeed, penetrance for thrombotic risk is compounded among carriers that are exposed to clinical risk factors such as estrogen therapy (37, 40, 55, 73, 74, 80, 93, 94, 100). These findings suggest that identification of genetic variation, single nucleotide polymorphisms (SNPs), or haplotypes within these pathways for coagulation and immunity with those related to hormonal status and diagnostic genotyping (or haplotyping), could better stratify patients exposed to clinical risk factors into high- and low-thrombotic risk, identify unexposed patients at risk for development of cardiovascular disease, and better target prophylaxis.

Although the genetic variant, factor V Leiden, is associated with increased relative risk for thrombosis with estrogen treatment (31, 47, 72), estrogenic-associated thrombosis occurs in individuals without this genetic variant (6, 32, 75a). Evidence linking genetic variation in other hemostatic factors such as fibrinogen, factor VII, and prothrombin with thrombosis is equivocal (11). However, as treatment with selective estrogen receptor modulators tamoxifen and raloxifene also increases risk of venous thrombosis, ligand-bound estrogen receptors may be causal to this increased risk (6). To date, other trials/studies that have evaluated effects of hormone treatment on progression/outcomes of cardiovascular disease have not evaluated estrogen receptor polymorphism with the genetic component of proteins of the coagulation cascade with procoagulant activity of the blood. Therefore, this study was undertaken to evaluate genetic polymorphisms in estrogen receptors and pathways of genes within the anticoagulant, procoagulant, fibrinolytic, or innate immunity pathways with subclinical cardiovascular disease in women enrolled in the Kronos Early Estrogen Prevention Study (KEEPS). KEEPS is a multicentered randomized, placebo-controlled, double-blinded, prospective trial of low-dose hormone therapy (oral or transdermal) in recently randomized, placebo-controlled, double-blinded, prospective trial of Estrogen Prevention Study (KEEPS). KEEPS is a multicentered cardiovascular disease in women enrolled in the Kronos Early pathways of genes within the anticoagulant, procoagulant, to evaluate genetic polymorphisms in estrogen receptors and anticoagulant activity of the blood. Therefore, this study was undertaken to evaluate genetic polymorphisms in estrogen receptors and pathways of genes within the anticoagulant, procoagulant, fibrinolytic, or innate immunity pathways with subclinical cardiovascular disease in women enrolled in the Kronos Early Estrogen Prevention Study (KEEPS). KEEPS is a multicentered randomized, placebo-controlled, double-blinded, prospective trial of low-dose hormone therapy (oral or transdermal) in recently menopausal women on progression of subclinical atherosclerosis defined by changes in carotid intima-media thickening and coronary arterial calcification over 4 years (27).

Focusing on genotyping for estrogen receptor polymorphisms and establishing risk for hormone therapy carry immediate and practical implications for decisions as to whether or not to use estrogen treatments but also for decisions regarding what type of estrogen treatment is most appropriate to reduce risk of adverse events (46, 75a, 77, 78, 95).

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. KEEPS inclusion criteria were age 42–58 yr, having at least 6 mo but no more than 36 mo of amenorrhea from last spontaneous menses occurring after age 40 yr, follicle stimulating hormone values ≥35 ng/mL and/or estradiol levels <35 pg/mL, and good general health. Women were excluded from KEEPS if they had a history of clinically defined cardiovascular disease; were current smokers (>10 cigarettes (half pack)/day by self-report); had a coronary artery calcium (CAC) score ≥50 Agatston units (AU), body mass index (BMI) >35 kg/m²; or if they had dyslipidemia (low-density lipoprotein cholesterol >190 mg/dl), hypertriglyceridemia (triglycerides, >400 mg/dl), 17β-estradiol >40 mg/dl, uncontrolled hypertension (systolic blood pressure >150 mmHg and/or diastolic blood pressure >95 mmHg), or fasting blood glucose >126 mg/dl (27, 60).

Enrollment began in July 2005 and was completed in June 2008 with 728 women meeting inclusion criteria and randomized to treatment: oral conjugated equine estrogen (Premarin, 0.45 mg/day), transdermal 17β-estradiol (via skin patch, Climara, 50 µg/day) both with progesterone (oral Premomet, 200 mg/day) for the first 12 days of the month, or placebo group (inactive pill/patch) (60). Of women randomized to treatment, 684 consented to allow analysis of their DNA. Clinical and lifestyle parameters used in this study were collected at screening and baseline visits prior to randomization to treatment.

The institutional review boards of all participating institutions approved this study, and all women signed informed consent.

Blood collection and preparation of DNA. All blood specimens were collected following an overnight fast and then frozen at −70°C on site until they were either processed locally or sent to the Kronos Science Laboratory (Phoenix, AZ) for storage or assays. Complete blood count and chemistry panel were performed at the clinical laboratories at each recruiting center. 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). SNPs for the custom 16,720 bead Illumina Infinium [13,229 SNPs including 492 ancestry informative markers (AIMs)] include candidate genes selected from electronic databases that annotate the anticoagulant, procoagulant, fibrinolytic, and/or innate immunity pathways with focus on platelet, monocyte, neutrophil and endothelial cell agonists, receptors, ligands, signal transduction and adhesion molecules, granule content and effectors; plasma proteases and inhibitors; matrix metalloproteases; inflammatory cytokines and receptors; estrogen, progesterone and androgen receptors, co-regulators and enzymes related to metabolism of estrogen; and enzymes associated metabolism of catechols, homocysteine, thromboxane A2, prostacyclin and 3-hydroxy-3-methylglutaryl coenzyme A reductase (30). Furthermore, the Estrogen Response Element Database (ERE DB) and Hormone Receptor Target Binding Loci Data Base (HRTDLDB) were checked for the genes of interest using the official gene symbol as well as official gene name aliases on the respective chromosomes.

Genotyping and quality control. Leukocyte genomic DNA was extracted, quantified, and diluted to the appropriate concentration for Illumina Infinium iSelect genotyping on all samples collected. Controls included 2% sample replicates and a trio from the Centre d’Etude du Polymorphisme Humain (CEPH) for quality control. In addition, DNA sample addresses were randomly assigned across both the 96-well plate as well as the 12-address iSelect BeadChip to avoid potential plate and chip effects, respectively. Genotyping results from high-quality control DNA (SNP call rate ≥95%) were used to generate a cluster algorithm.

Anthropometric and lifestyle exposures. Baseline smoking status (current use yes/no) and race/ethnicity were obtained through self-reported questionnaire data. The height (cm) and weight (kg) of
participants, wearing light clothing and without shoes, were measured with stadiometers and calibrated balance-beam scales, respectively. BMI was calculated as weight in kilograms divided by the square of height in meters. Waist circumference (cm) was measured with a nonstretchable tape after a normal expiration at the smallest horizontal circumference between the ribs and iliac crest. Blood pressure was measured in the right arm in the seated position, after at least a 5 min rest, and averaged across two readings. Women ranked their menopausal symptoms (hot flashes, night sweats, vaginal dryness, dyspareunia, palpitations, insomnia, depression, mood swings, and irritability) on a score of 1–3 as either none (0), mild (1), moderate (2), or severe (3). A weighted sum of the scores for menopausal symptoms was calculated based on the following formula: 3(hot flashes) + 2(dyspareunia + vaginal dryness + night sweats) + mood swings + insomnia + 0.5(palpitations + depression + irritability).

**Measures of atherosclerosis.** Carotid artery intima-media thickness (CIMT) was measured via B-mode ultrasound at each study center by certified ultrasound technicians trained at the Core Imaging and Reading Center (CIRC; PI: Dr. Howard Hodis, USC) to perform a standard acquisition sequence (patents 2005, 2006, 2011). Image acquisition procedures were optimized for minimal measurement variability (33). The ultrasound power, echo detector gain, and dynamic range were recorded to establish identical conditions for serial examinations. All instruments were high-resolution imagers with a multiarray 7.5 MHz probe. Electrocardiogram and ultrasound images were simultaneously recorded. All images were evaluated at the CIRC by an experienced investigator with in-house developed automated computerized edge-detection software (patents 2005, 2006, 2011) (33, 81).

CIMT was measured on nonenhanced cardiac computed tomography scans. CIMT was defined as a plaque of at least 3 contiguous pixels (area $1.02 \text{ mm}^2$) with a density of $\geq 130 \text{ HU}$. A total CIMT score was determined by summing the individual Agatston scores from each of four anatomic sites (left main, left anterior descending, circumflex, and right coronary). A single experienced investigator, blinded to the subject identity, interpreted all the scans using commercially available software (TeraRecon, Foster City, CA) (12, 36, 62, 76).

**Statistical analysis.** The analysis consists of two primary outcomes, CIMT as a continuous measure and CAC as a binary measure. Descriptive statistics were used to summarize the cohort variables including means with standard deviations (SD) and medians with interquartile ranges (Q1, Q3) for all continuous parameters. Since the distribution of CIMT is skewed, this variable was log-transformed. We used three HapMap 2 populations (CEU: Utah residents from the CEPH; YRI: Yoruba from Ibadan, Nigeria; and CHB/JPT: Han Chinese from Beijing, China, and Japanese from Tokyo, Japan) to characterize the set of allele frequencies at each locus of the 492 AIMs (79). To identify the population structure of KEEPS we used these 492 AIMs and the program STRUCTURE (66). Within the KEEPS sample most were Caucasian, and we used percent CEU from the STRUCTURE program to determine the proportion of European ancestry within each individual. STRUCTURE allows for population admixture and assigns individuals in the sample of interest (the KEEPS sample) population proportions determined by individual allele frequencies. The technique assumes the loci are unlinked, and it assumes Hardy-Weinberg equilibrium within the populations.

Prior to the single SNP analysis, we identified for each outcome the following nongenetic risk factors (demographic and clinical variables). The proportion of European ancestry as determined by STRUCTURE, self-reported “white,” age in years at study enrollment, low-density lipoprotein cholesterol level (mg/dl), high-density lipoprotein cholesterol level (mg/dl), triglyceride level (mg/dl), fasting glucose (mg/dl), hypertension diagnosis (by history on phone screen), weighted algorithm of menopausal scores, waist circumference (cm), pulse pressure [mean of systolic blood pressure at pre-enrollment, baseline visit (visit 0) and enrollment, randomization visit (visit 1) minus mean of diastolic blood pressure at visits 0 and 1 in mmHg], and current smoking status (yes/no). The clinical variables were regressed against the natural log of CIMT using linear regression. Two analyses were performed. First, all 12 variables were jointly regressed against log CIMT. Second, a stepwise linear regression was performed using alpha equal 0.10 to enter or leave. Model residuals were examined to see that modeling assumptions were met. We considered only variables with $P \leq 0.0001$ significant for use as adjusting variables when examining the association with the genotyped SNPs. Proportion of European ancestry, age at enrollment, and pulse pressure were significant in the stepwise linear regression and were used as adjusting variables in subsequent analysis of log-transformed CIMT.

Similarly, the relationship between demographic and clinical variables and CAC was examined in an unconditional logistic regression. The same demographic and clinical variables were included in models of CAC as listed above. As above, all 12 variables were jointly examined, and then, subsequently, a stepwise logistic regression was performed. Model assumptions were checked; only model variables with $P \leq 0.01$ were used as adjusting variables. Only waist circumference was significantly associated with CAC.

The association between each SNP and log-transformed CIMT was tested by a linear regression model with additive genetic effect adjusting for age, proportion of European ancestry, and pulse pressure and for CAC by unconditional logistic regression with additive genetic effect adjusting for waist circumference. All above analyses were done with SAS software version 9.1.

These analyses were corrected for multiple comparisons with an extension of false discovery rates (8). The false discovery rate is an analog measure of the $P$ value that takes into account the number of statistical tests and estimates the expected proportion of false positive tests incurred when a particular SNP is significant. All analyses were performed using PLINK v 1.07 (67).

**RESULTS**

From the 684 DNA samples, 74 were removed for the following reasons: HapMap CEPH genotype controls (18), misidentified (16), call rates <0.95 (8), relatedness to another sample (5), duplicate samples (26), ineligible sample (1). Due to incomplete phenotypic data, 11 samples were removed from the CIMT analysis and 10 from the CAC analysis. From the 13,229 SNPs, only 11,955 were used for association analysis. A total of 1,274 SNPs were excluded from analysis due to call rate <0.95 (714), monomorphic (no minor alleles, 68), and ancestry informative markers (492).

Population stratification from the individual DNA samples using three HapMap Phase 11 populations identified the majority of participants to be of Central European ancestry (Fig. 1 and Table 1). Individuals reporting themselves as Hispanic, Black, or Asian were of mixed genetic ancestry (Table 1).

Collective phenotypic characteristics of the 610 women for whom SNP analyses were performed are provided in Table 2. The mean and standard deviation for CIMT for these genotyped individuals were 0.72 and 0.09 mm, respectively (median $= 0.70 \text{ mm}$, range 0.53–1.17 mm). Covariates that significantly associated positively with the log-transformed CIMT were age at study enrollment and pulse pressure, whereas proportion of European ancestry (CEU) per subject associated negatively with CIMT (Table 3). Each unit of CEU proportion decreased the log-transformed CIMT, and each unit of age at study enrollment and pulse pressure increased the log-transformed CIMT. The adjusted R-square of these three variables or the contribution to overall variance of log CIMT is low (8.5%). To avoid potential false positive SNPs we adjusted...
Table 1. Proportion of ancestry for 4 main races (collapsing all the Asians under 1 category)

<table>
<thead>
<tr>
<th>Reported Race</th>
<th>n</th>
<th>CHB/JPT</th>
<th>CEU</th>
<th>YRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No answer/other</td>
<td>40</td>
<td>0.019</td>
<td>0.938</td>
<td>0.043</td>
</tr>
<tr>
<td>Asian</td>
<td>17</td>
<td>0.723</td>
<td>0.256</td>
<td>0.021</td>
</tr>
<tr>
<td>Black</td>
<td>43</td>
<td>0.013</td>
<td>0.181</td>
<td>0.807</td>
</tr>
<tr>
<td>White</td>
<td>466</td>
<td>0.008</td>
<td>0.986</td>
<td>0.007</td>
</tr>
<tr>
<td>Hispanic</td>
<td>44</td>
<td>0.164</td>
<td>0.669</td>
<td>0.167</td>
</tr>
</tbody>
</table>

CEU, Utah residents from the Centre d’Etude du Polymorphism Humain; YRI, Yoruba from Ibadan, Nigeria; CHB/JPT, Han Chinese from Beijing, China, and Japanese from Tokyo, Japan.

Table 2. Characteristics of genotyped participants in KEEPS

<table>
<thead>
<tr>
<th>Covariate</th>
<th>n</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at menopause, yr</td>
<td>609</td>
<td>51.3 ± 2.5</td>
<td>42.2–57.5</td>
</tr>
<tr>
<td>Time past menopause, yr</td>
<td>610</td>
<td>1.43 ± 0.72</td>
<td>0.5–3.0</td>
</tr>
<tr>
<td>Weighted menopausal symptom score</td>
<td>610</td>
<td>12.7 ± 6.8</td>
<td>0.00–33.0</td>
</tr>
<tr>
<td>Smoking status (current cigarette smoker)</td>
<td>610</td>
<td>39 (6.4%)</td>
<td></td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>602</td>
<td>84.9 ± 11.8</td>
<td>57.2–120.0</td>
</tr>
<tr>
<td>Body mass index, kg/mm²</td>
<td>610</td>
<td>26.3 ± 4.3</td>
<td>16.9–40.0</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>610</td>
<td>119.0 ± 15.1</td>
<td>82.0–189.0</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>610</td>
<td>74.9 ± 9.3</td>
<td>50.0–113.0</td>
</tr>
<tr>
<td>Fasting blood glucose, mg/dl</td>
<td>610</td>
<td>89.4 ± 9.8</td>
<td>55.0–126.0</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>610</td>
<td>65.4 ± 17.4</td>
<td>24.0–129.0</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl</td>
<td>610</td>
<td>128.7 ± 30.1</td>
<td>11.0–194.0</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>610</td>
<td>89.7 ± 50.2</td>
<td>7.0–374.0</td>
</tr>
</tbody>
</table>

Data are shown as means ± SD, n, number of genotyped individuals; KEEPS, Kronos Early Estrogen Prevention Study.

However, none of the SNPs were significant after correcting for multiple testing.

Searches of both the ERE DB and the HRTDL DB indicated only one of the six genes of interest, the SERPINA 1 gene, was registered with an estrogen receptor response element.

Table 3. Significant independent covariates for CIMT and CAC used in the regression analyses

<table>
<thead>
<tr>
<th>Covariate</th>
<th>β-Coefficient (SE)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIMT</td>
<td>−0.088 (0.018)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Proportion of European ancestry</td>
<td>0.0076 (0.0019)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age at enrollment</td>
<td>0.0017 (0.0004)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pulse pressure, mmHg</td>
<td>0.028 (0.0098)</td>
<td>0.0044</td>
</tr>
</tbody>
</table>

CIMT, carotid artery intima-media thickness; CAC, coronary artery calcification. *Odds ratio = 1.03.

DISCUSSION

In this targeted candidate genetic analysis to evaluate potential effects of MHT on progression of cardiovascular disease and thrombotic risk as complex genetic traits, genetic variation only within genes of the innate and humoral immunity pathways and not of the anticoagulant, procoagulant, or fibrinolytic pathways associated with subclinical cardiovascular disease defined by CIMT and CAC. Although statistical significance of some associations was lost after correcting for multiple testing, the contribution of the identified genes will be discussed relative to current investigations of single trait analyses.

Contrary to our hypothesis, neither CIMT nor CAC showed a relationship to polymorphisms of either estrogen receptor alpha or beta. However, women were estrogen deplete for a mean of 1.5 yr at the time of testing. It remains to be determined whether or not polymorphisms in estrogen receptors or in enzymes involved in estrogen metabolism will associate with changes in either CIMT or CAC after 4 yr of MHT. Genetic variance in both estrogen receptors and enzymes involved in steroid metabolism associated with cardiovascular risk factors in women of four different ethnic groups in the Study of Women Across the Nation (SWAN) (85, 87, 88). Although some cardiovascular risk factors segregated by race/ethnicity in the SWAN, no analyses were performed for AIMs, and differences among race/ethnic groups were not
consistently observed and may have reflected life style, cultural, and/or environmental differences (57, 87). In the present study, CIMT was negatively associated with proportion of European ancestry.

By design, women eligible for randomization to KEEPS had low cardiovascular risk profiles (BMI, fasting serum blood glucose, triglycerides, HDL-C, LDL-C, and blood pressure within normative ranges). However, as it has been reported in studies of other populations, CIMT correlated with age and blood pressure (pulse pressure in the current study) (39, 56, 70, 82).

Activation of inflammatory processes contributes to progression of cardiovascular disease (50). Therefore, it might be expected for variants in genes encoding proteins within the inflammatory pathway to associate with subclinical cardiovascular disease measured as CIMT and CAC. However, the differential association of SNPs with CIMT and CAC suggests heterogeneous mechanisms for development of arterial wall thickening within carotid arteries and calcification of the coronary arteries. The concept of distinct risk factors and processes for development of disease based on the anatomical origin of the blood vessel is supported by other studies (34, 35, 38, 97).

In the present study, an SNP in the MAP4K4 gene, a serine/threonine kinase mediating TNF-α signaling, and the IL5 gene, encoding a cytokine that stimulates growth and differentiation of B cells (humoral immunity) and eosinophils, associated positively with CIMT. Both of these pathways have been implicated in development of vascular lesions in experimental animals and are regulated by estrogen (2, 19, 45, 54). MAP kinase is one of the signaling pathways activated by binding of pathogen-associated molecular patterns to the toll-like receptor 4 (TLR4)-CD14 dimer on the surface of macrophages and considered a component of innate immunity. Estrogenic regulation of these genes would alter expression of modified gene product.

Alternatively, two SNPs within the CCL5 (RANTES) gene associated negatively with CIMT. Activation of CCL5 and its receptor accelerates development of atherosclerosis in mice models of the disease (41, 43). RANTES is also considered a component of the humoral inflammatory pathway (51), indicating that direct and indirect interactions among components of the hemostasis and inflammatory pathways are complex and components of each cannot be considered in isolation. Whether these SNPs result in a defect in RANTES signaling remains to be tested in isolated human tissues. Furthermore, the product of

Table 4. Significant SNPs associated with CIMT

<table>
<thead>
<tr>
<th>SNP</th>
<th>Gene Name</th>
<th>Chr</th>
<th>Position, base pair</th>
<th>Risk Allele</th>
<th>n</th>
<th>Beta</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2236935</td>
<td>MAP4K4</td>
<td>2</td>
<td>101810474</td>
<td>G</td>
<td>606</td>
<td>0.03697</td>
<td>2.36E-06</td>
</tr>
<tr>
<td>rs4796119</td>
<td>CCL5</td>
<td>17</td>
<td>31217201</td>
<td>G</td>
<td>607</td>
<td>-0.0427</td>
<td>3.59E-05</td>
</tr>
<tr>
<td>rs739718</td>
<td>IL5</td>
<td>5</td>
<td>131900972</td>
<td>G</td>
<td>607</td>
<td>0.05122</td>
<td>5.02E-05</td>
</tr>
<tr>
<td>rs2291299</td>
<td>CCL5</td>
<td>17</td>
<td>31215519</td>
<td>G</td>
<td>607</td>
<td>-0.03179</td>
<td>5.59E-05</td>
</tr>
</tbody>
</table>

SNP, single nucleotide polymorphism. *Uncorrected P.
this SNP may be interacting with another gene that has other direct or indirect effects on development of CIMT. Previously, CAPN10-haplotype on chromosome 2 was also found through targeted gene studies, as well as through genome-wide association study (GWAS) to have strong association with CIMT (22, 23, 96).

Consistent with other studies, waist circumference associated with a positive CAC score in KEEPS participants (21, 42). When we adjusted for waist circumference, two SNPs associated positively with CAC. One SNP was found on the gene for IRAK2, which encodes a protein affecting transcriptional regulation, mRNA stability, and the IL-1-induced upregulation of NF-κB, thus linking innate immunity with development of CAC.

The other SNP positively associated with CAC was on the SERPINA1 gene (14q32.13), which encodes the protease inhibitor alpha 1-antitrypsin that breaks down elastase. This SNP most frequently associates with lung fibrosis. Although this defect could affect elastase in arterial tissue leading to arterial stiffening, the relationship of this SNP to development calcific processes remains to be explored. This gene was found to have an estrogen response element in the promoter region. In the Framingham Heart Study, the African Americans in the Hypertension Genetic Epidemiology Network study, and a study in Dominican Republican families, quantitative trait loci for CIMT associated with arterial stiffness also were mapped to chromosome 14 (97). Of interest is that SNP (rs7152362) resides on chromosome 14q32.12. The relationship of that SNP to calcification processes also remains to be explored.

An SNP of the ABO gene that negatively correlated with CAC encodes proteins affecting posttranslational modification of proteins, for example, von Willebrand factor, a protein carrier for coagulation factor VIII. In a case-controlled study including mostly pre- or postmenopausal women, a case-controlled study including both men and women, and a GWAS, risk of venous thrombosis was associated with a non-O blood type (29, 30, 99). Estrogen is known to modulate both mRNA stability and posttranslational modification of proteins (61). Therefore, it will be interesting to determine whether these SNPs for IRAK2 and ABO remain associated with CAC in women treated with estrogen. After we corrected for multiple comparisons, none of these SNPs remained significantly asso-

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**Table 5. Description of significant SNPs associated with CAC**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Gene Name</th>
<th>Chr</th>
<th>Position, base pair</th>
<th>Risk Allele</th>
<th>n</th>
<th>Odds Ratio</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs11465886</td>
<td>IRAK2</td>
<td>3</td>
<td>10225783</td>
<td>G</td>
<td>599</td>
<td>3.909</td>
<td>0.000110</td>
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<tr>
<td>rs17751769</td>
<td>SERPINA1</td>
<td>14</td>
<td>93926410</td>
<td>A</td>
<td>575</td>
<td>1.955</td>
<td>0.000242</td>
</tr>
<tr>
<td>rs630014</td>
<td>ABO</td>
<td>9</td>
<td>135139543</td>
<td>A</td>
<td>599</td>
<td>0.508</td>
<td>0.000251</td>
</tr>
<tr>
<td>rs702689</td>
<td>MAP3K1</td>
<td>5</td>
<td>56213200</td>
<td>G</td>
<td>595</td>
<td>1.784</td>
<td>0.000869</td>
</tr>
<tr>
<td>rs1610517</td>
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<td>14</td>
<td>93928109</td>
<td>G</td>
<td>599</td>
<td>1.834</td>
<td>0.000949</td>
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<tr>
<td>rs832573</td>
<td>MAP3K1</td>
<td>5</td>
<td>56195335</td>
<td>A</td>
<td>599</td>
<td>1.766</td>
<td>0.000995</td>
</tr>
</tbody>
</table>

*Uncorrected P.
associated with CAC, reflecting perhaps the low level of calcification (<50 AU in KEEPS), which is considered nonclinically relevant or inadequate power to detect an effect as only 80 women eligible for the study had a positive CAC score.

Other genome-wide and targeted studies have identified associations of several other genetic variants with CIMIT and CAC including genes encoding proteins for lipid metabolism, angiotensin-converting enzyme, TLR4 (innate immunity), and bone formation (3, 16, 25, 63, 64, 91). The value of these analyses in cardiovascular risk prediction in humans is inconsistent and does not necessarily provide value beyond those of more conventional risk phenotypic characteristics (92, 97). These inconsistencies may result from associations with events reflecting advanced disease rather than determinates of subclinical disease, analysis adjusted for sex rather than dichotomized by sex, not accounting for hormonal status, and influences of ancestry-related genotypes (14, 15, 24, 92, 97). Strengths of the current investigation lie in a targeted genetic approach in healthy individuals in which it will be possible to determine longitudinal assessment of these genetic associations with disease progression following a single intervention (MHT) in women completing the KEEPS trial.

In summary, in a targeted gene analysis of healthy, recently menopausal women, distinct genetic variants associated with measures of subclinical cardiovascular disease. Estrogen is known to modulate some of the processes regulated by the identified genes. Therefore, it will be possible to evaluate how MHTs affect the phenotypic expression of these modulators of disease progression in KEEPS participants randomized to MHT completing the study.

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86 SNPs AND SUBCLINICAL ATHEROSCLEROSIS IN WOMEN OF KEEPS


SNPs and Subclinical Atherosclerosis in Women of Keeps