

# Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart  
Association®



*Learn and Live* SM

## Association of the Vitamin D Metabolism Gene CYP24A1 With Coronary Artery Calcification

Haiqing Shen, Lawrence F. Bielak, Jane F. Ferguson, Elizabeth A. Streeten, Laura M. Yerges-Armstrong, Jie Liu, Wendy Post, Jeffery R. O'Connell, James E. Hixson, Sharon L.R. Kardia, Yan V. Sun, Min A. Jhun, Xuexia Wang, Nehal N. Mehta, Mingyao Li, Daniel L. Koller, Hakan Hakonarson, Brendan J. Keating, Daniel J. Rader, Alan R. Shuldiner, Patricia A. Peyser, Muredach P. Reilly and Braxton D. Mitchell

*Arterioscler Thromb Vasc Biol* 2010;30:2648-2654; originally published online Sep 16, 2010;

DOI: 10.1161/ATVBAHA.110.211805

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

Copyright © 2010 American Heart Association. All rights reserved. Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://atvb.ahajournals.org/cgi/content/full/30/12/2648>

Data Supplement (unedited) at:

<http://atvb.ahajournals.org/cgi/content/full/ATVBAHA.110.211805/DC1>

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at

<http://atvb.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail:

[journalpermissions@lww.com](mailto:journalpermissions@lww.com)

Reprints: Information about reprints can be found online at

<http://www.lww.com/reprints>

# Association of the Vitamin D Metabolism Gene *CYP24A1* With Coronary Artery Calcification

Haiqing Shen, Lawrence F. Bielak, Jane F. Ferguson, Elizabeth A. Streeten, Laura M. Yerges-Armstrong, Jie Liu, Wendy Post, Jeffery R. O'Connell, James E. Hixson, Sharon L.R. Kardia, Yan V. Sun, Min A. Jhun, Xuexia Wang, Nehal N. Mehta, Mingyao Li, Daniel L. Koller, Hakan Hakonarson, Brendan J. Keating, Daniel J. Rader, Alan R. Shuldiner, Patricia A. Peyser, Muredach P. Reilly, Braxton D. Mitchell

**Objective**—The vitamin D endocrine system is essential for calcium homeostasis, and low levels of vitamin D metabolites have been associated with cardiovascular disease risk. We hypothesized that DNA sequence variation in genes regulating vitamin D metabolism and signaling pathways might influence variation in coronary artery calcification (CAC).

**Methods and Results**—We genotyped single-nucleotide polymorphisms (SNPs) in *GC*, *CYP27B1*, *CYP24A1*, and *VDR* and tested their association with CAC quantity, as measured by electron beam computed tomography. Initial association studies were carried out in a discovery sample comprising 697 Amish subjects, and SNPs nominally associated with CAC quantity (4 SNPs in *CYP24A1*,  $P=0.008$  to  $0.00003$ ) were then tested for association with CAC quantity in 2 independent cohorts of subjects of white European ancestry (Genetic Epidemiology Network of Arteriopathy study [ $n=916$ ] and the Penn Coronary Artery Calcification sample [ $n=2061$ ]). One of the 4 SNPs, rs2762939, was associated with CAC quantity in both the Genetic Epidemiology Network of Arteriopathy ( $P=0.007$ ) and Penn Coronary Artery Calcification ( $P=0.01$ ) studies. In all 3 populations, the rs2762939 C allele was associated with lower CAC quantity. Metaanalysis for the association of this SNP with CAC quantity across all 3 studies yielded a  $P$  value of  $2.9 \times 10^{-6}$ .

**Conclusion**—A common SNP in the *CYP24A1* gene was associated with CAC quantity in 3 independent populations. This result suggests a role for vitamin D metabolism in the development of CAC quantity. (*Arterioscler Thromb Vasc Biol.* 2010;30:2648-2654.)

**Key Words:** calcification ■ coronary artery disease ■ epidemiology ■ gene mutations ■ vitamin D metabolism

Coronary artery calcification (CAC) is a measure of coronary subclinical atherosclerosis that predicts risk for cardiovascular disease (CVD) in the general population.<sup>1</sup> The potential role of circulating levels of vitamin D in influencing the initiation or progression of CAC is of great clinical interest given the large proportion of the population deficient in vitamin D<sup>2</sup>, the fact that the vitamin D endocrine system is essential for calcium homeostasis, and that low vitamin D levels have been associated with risk for CVD.<sup>2</sup> However, circulating levels of vitamin D metabolites have not been consistently associated with CAC, although causal relation-

ships may not be apparent from simple cross-sectional studies.

## See accompanying article on page 2329

The effects of vitamin D are myriad, including enhancement of cellular differentiation and immunomodulation, in addition to the well-known effects on calcium homeostasis.<sup>3</sup> The effects of vitamin D are mediated by activation of the nuclear vitamin D receptor (VDR), which regulates transcription of a large number of genes in many tissues. The metabolism of vitamin D, providing active  $1,25(\text{OH})_2\text{D}$  to the

Received on: June 24, 2010; final version accepted on: September 7, 2010.

From the Division of Endocrinology, Diabetes and Nutrition, University of Maryland School of Medicine, Baltimore, Md (H.S., E.A.S., L.M.Y.-A., J.L., J.R.O., A.R.S., B.D.M.); Department of Epidemiology, University of Michigan School of Public Health, Ann Arbor, Mich (L.F.B., S.L.R.K., Y.V.S., M.A.J., P.A.P.); The Cardiovascular Institute (J.F.F., N.N.M., B.J.K., D.J.R., M.P.R.), Biostatistics and Epidemiology (X.W., M.L.), and The Institute for Translational Medicine and Therapeutics, School of Medicine (N.N.M., M.P.R.), University of Pennsylvania, Philadelphia, Pa; Geriatric Research and Education Clinical Center, Department of Veterans Affairs Medical Center, Baltimore, Md (E.A.S., A.R.S.); Division of Cardiology, School of Medicine (W.P.) and Department of Epidemiology, Bloomberg School of Public Health (W.P.), Johns Hopkins University, Baltimore, Md; Human Genetic Center, School of Public Health, The University of Texas Health Science Center at Houston, Houston, Texas (J.E.H.); Department of Medical and Molecular Genetics, School of Medicine, Indiana University, Indianapolis, Ind (D.L.K.); Center for Applied Genomics, Children's Hospital of Philadelphia, Philadelphia, PA (H.H.).

Correspondence to Braxton D. Mitchell, PhD, Division of Endocrinology, Diabetes and Nutrition, University of Maryland School of Medicine, 660 West Redwood St, Rm 492, Baltimore, MD 21201. E-mail [bmitchel@medicine.umaryland.edu](mailto:bmitchel@medicine.umaryland.edu)

© 2010 American Heart Association, Inc.

*Arterioscler Thromb Vasc Biol* is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.110.211805

VDR, requires the involvement of several key proteins in a tightly regulated process. Vitamin D (made in the skin keratinocytes in response to UVB light or from exogenous supplements) is sequentially hydroxylated by the mitochondrial cytochrome P450 enzymes 25-hydroxylase (the product of *CYP2R1*) in the liver and 1- $\alpha$  hydroxylase (the product of *CYP27B1*) in the kidney (among other tissues) into the active metabolite 1,25(OH)<sub>2</sub>D. After 1,25(OH)<sub>2</sub>D binds to the ubiquitous VDR, the retinoid X receptor<sup>4</sup> is recruited, forming a heterodimer complex that regulates gene transcription by interacting with vitamin D response elements in the promoter region of genes. Vitamin D metabolites are transported through the circulation by vitamin D binding protein (GC).

Concentrations of 1,25(OH)<sub>2</sub>D are tightly regulated by both 1-hydroxylase and the catabolic enzyme 24-hydroxylase (gene *CYP24A1*). *CYP24A1* is induced by both 1,25(OH)<sub>2</sub>D and 25(OH)D and is one of the most highly inducible genes in humans, capable of increasing its transcription by 20 000-fold.<sup>5</sup> The high inducibility of *CYP24A1* is likely to be a critical factor in the large therapeutic window of vitamin D.

Thus, the key genes involved in vitamin D metabolism pathway include *CYP2R1*, *CYP27B1*, *CYP24A1*, *VDR*, and *GC*. We hypothesized that DNA sequence variation in these genes and their levels might influence CAC quantity variation. To test this hypothesis, we obtained genotypes of single-nucleotide polymorphisms (SNPs) in 4 available genes (*GC*, *CYP27B1*, *CYP24A1*, and *VDR*) of the 5 major genes involved in the regulation of vitamin D levels and tested their association with CAC quantity. Initial association studies were carried out in a discovery sample comprising 697 Amish subjects, and SNPs nominally associated with CAC quantity in this cohort were then tested for association with CAC quantity in 2 independent cohorts of subjects who were also of white European ancestry (the Genetic Epidemiology Network of Arteriopathy [GENOA] Study and the Penn Coronary Artery Calcification [PennCAC] sample).

## Populations and Methods

### Discovery Population (Amish)

The discovery sample comprised participants in the Amish Family Calcification Study (AFCS), a community-based study initiated in 2001 to identify the joint determinants of bone mineral density and vascular calcification. Details of the recruitment procedures have been published previously.<sup>6</sup> Many related individuals were recruited (indeed, all Amish are related), and recruitment efforts were made without regard to CVD health status in this generally healthy population. Because vascular calcification occurs infrequently in young adulthood, only men aged 30 years and older or women aged 40 years or older were recruited into the AFCS. The protocol was approved by the Institutional Review Board of the University of Maryland and other participating institutions. Informed consent, including permission to contact relatives, was obtained before participation.

All AFCS participants underwent a detailed medical history interview and assessment of potential risk factors for CVD at the Amish Research Clinic in Strasburg, Pennsylvania. Physical examinations were conducted in the early morning following an overnight fast. Blood samples were obtained for biochemical measurements and DNA extraction.

Images of the coronary arteries were obtained by electron beam computed tomography using an Imatron C-150 scanner. The protocol included 30 to 40 3-mm contiguous transverse slices between the

aortic root and the apex of the heart, gated to 80% of the R-to-R interval (the cycle between 2 consecutive R waves) and obtained during a single breath hold. The extent of calcification in the thoracic aorta was assessed by scanning between the superior aspect of the aortic arch and the superior pole of the kidney at 6-mm intervals. The presence of calcification was defined as a density >130 Hounsfield units with area >1 mm<sup>2</sup>. CAC was quantified using the Agatston method, which incorporates both density and area.<sup>7</sup> The sum of the scores in the left main, left anterior descending, circumflex, and right coronary arteries was considered the CAC score. All scans were scored by a single experienced cardiologist.

A total of 786 AFCS study participants were genotyped using the HumanCVD BeadChip V2 (Illumina, Inc., San Diego, Calif), which includes  $\approx$ 50 000 SNPs within  $\approx$ 2000 CVD candidate genes.<sup>8</sup> *GC*, *CYP27B1*, and *VDR* were considered in the chip design as group 1 genes, with denser SNP coverage (inclusive of the intronic and exonic untranslated regions and 5 kb of the proximal promoter regions), whereas *CYP24A1* was considered a group 2 gene, with sparser SNP coverage (inclusive of intronic, exonic, and flanking untranslated regions). In the chip design, tag SNPs were selected from group 1 genes to capture known variation across all of the 4 representative HapMap populations and SeattleSNPs (where available) for SNPs having minor allele frequency >0.02 and an  $r^2$  of at least 0.8. Tag SNPs included in group 2 genes were designed to capture known variation for SNPs having minor allele frequency >0.05 and an  $r^2$  of at least 0.5.<sup>8</sup> Carriers for the known monogenic mutation *R3500Q* in Apolipoprotein B-100 (n=81),<sup>9</sup> responsible for familial defective apob-100, were excluded from the analysis. *R3500Q* has been associated with CAC quantity in the Amish.

The HumanCVD BeadChip included 11, 13, 21, and 116 SNPs in the *GC*, *CYP27B1*, *CYP24A1*, and *VDR* genes, respectively. The initial genotype calls were generated with the cluster file provided by Illumina BeadStudio software. Individuals with call rates less than 95% were excluded (n=8). SNPs with a low call rate (<95%) were excluded. Of successfully genotyped SNPs, 3, 10, 3, and 47 were monomorphic in the Amish across *GC*, *CYP27B1*, *CYP24A1*, and *VDR*, respectively, and 5 SNPs (3 from *CYP24A1* and 2 from *VDR*) were excluded because of low call rate. This left a total of 93 SNPs (8 in *GC*, 3 in *CYP27B1*, 15 in *CYP24A1*, and 67 in *VDR*) included in this analysis [SNP names are provided in Supplemental Table I, available online at <http://atvb.ahajournals.org>]. The observed distribution of genotypes was tested for deviation from Hardy-Weinberg equilibrium using the Pearson  $\chi^2$  test.

### Replication Populations

#### GENOA Study

The Family Blood Pressure Program, established by the National Heart, Lung, and Blood Institute in 1996, joined existing research networks that were investigating hypertension and CVD.<sup>10</sup> One of the 4 Family Blood Pressure Program networks is GENOA, which recruited sibships with hypertensive adults to investigate genetic contributions to hypertension and hypertension-related target organ damage. Sibships containing at least 2 individuals with clinically diagnosed essential hypertension before age 60 were recruited from Rochester, Minnesota. Participants were diagnosed with hypertension if they had either (1) a previous clinical diagnosis of hypertension by a physician with current antihypertensive treatment or (2) an average systolic blood pressure  $\geq$ 140 mm Hg or diastolic blood pressure  $\geq$ 90 mm Hg based on the second and third readings at the time of their clinic visit as stipulated by the Joint National Committee-7 guidelines.<sup>11</sup> Exclusion criteria were secondary hypertension, alcoholism or drug abuse, pregnancy, insulin-dependent diabetes mellitus, active malignancy, hypercalcemia, renal insufficiency (creatinine >1.3 mg/dL), and known metabolic bone disease other than osteoporosis. After identifying the hypertensive sibships, all members of the sibship were invited to participate regardless of their hypertension status.

Between December 2000 and February 2004, 1241 of the original GENOA study participants in the Rochester field center returned to undergo risk factor and target organ damage measurement. Individ-

uals with a history of coronary revascularization (n=83) were not eligible for measurement of CAC. After further excluding participants with a history of myocardial infarction (n=19), stroke (n=27), or positive angiogram (n=31); those who self-reported not being of European ancestry (n=2); and those missing risk factor data (n=2), there were 1077 GENOA participants with CAC and risk factor measures.

Participants were imaged with an Imatron C-150 electron beam computed tomography scanner (Imatron Inc., South San Francisco, Calif) as previously described.<sup>12</sup> CAC was defined as a hyperattenuating focus in a coronary artery area >1.0 mm<sup>2</sup> and having a density >130 Hounsfield Units (HU) with area >1 mm<sup>2</sup>. Quantity of CAC was defined as the total CAC score summed from the 4 major epicardial arteries using the method of Agatston et al.<sup>7</sup> The average CAC score from 2 sequential scans was used.

SNPs to be tested for replication (rs4809960, rs2296241, and rs2245153) had previously been genotyped in the GENOA Study on the Affymetrix Genome-Wide Human SNP Array 6.0 platform. All passed the overall quality control filters (eg, SNP call rate >95%). SNP imputation was performed using MACH, version 1.0.16 (<http://www.sph.umich.edu/csg/abecasis/MaCH>), based on the publicly available phased haplotypes from HapMap release 22, build 36, CEU population. Association analysis of GENOA SNP (rs2762939) was based on the imputed genotype dosage that ranges from 0 to 2, which is the expected number of effective alleles. The imputed genotype of rs2762939 had an imputation quality score of 0.80 and  $r^2$  of 0.62 with a genotyped SNP.

Of the 1077 GENOA participants with CAC and risk factor data, 916 had genotype data available for analyses. These 916 participants were in 422 sibships, including 115 singletons, 204 sibships of size 2, 64 sibships of size 3, 16 sibships of size 4, 13 sibships of size 5, 5 sibships of size 6, 1 sibship of size 7, 2 sibships of size 8, 1 sibship of size 9, and 1 sibship of size 10. The study was approved by the institutional review boards of all the participating institutions, and participants gave written informed consent.

### **PennCAC**

The PennCAC sample included subjects of white European ancestry recruited from 3 separate parallel studies: the Study of Inherited Risk of Coronary Atherosclerosis (n=799), the Penn Diabetes Heart Study (n=782), and the Philadelphia Area Metabolic Syndrome Network (n=480).<sup>13</sup> PennCAC samples were genotyped using the HumanCVD BeadChip V2 (Illumina, Inc.).

The Study of Inherited Risk of Coronary Atherosclerosis is a cross-sectional study of factors associated with CAC in a community-based sample of asymptomatic subjects and their families.<sup>14</sup> Subjects were healthy adults aged 30 to 75 years who had a family history of premature coronary artery disease. Subjects were excluded if they reported evidence of coronary artery disease on screening questionnaire, reported a history of diabetes mellitus, or had a serum creatinine level >3.0 mg/dL.

The Penn Diabetes Heart Study is an ongoing, cross-sectional community-based study of type 2 diabetic subjects without clinical evidence of coronary heart disease or overt chronic kidney disease.<sup>15</sup> Subjects were aged 35 to 75 years, had a clinical diagnosis of type 2 diabetes (defined as fasting blood glucose  $\geq$ 126 mg/dL, 2-hour postprandial glucose  $\geq$ 200 mg/dL, or use of oral hypoglycemic agents/insulin in a subject older than 40), and had a negative pregnancy test if female. Subjects were excluded if they had evidence of clinical coronary heart disease, a clinical diagnosis of type 1 diabetes (insulin use before age 35), a serum creatinine >2.5 mg/dL, or weight >300 lbs.

The Philadelphia Area Metabolic Syndrome Network is a cross-sectional study of patients with 1 or more metabolic syndrome risk factors. Philadelphia Area Metabolic Syndrome Network participants were recruited between 2004 and 2009 via the University of Pennsylvania Health System primary care providers, word of mouth in the community, and Penn health fairs for CVD risk factors. Subjects were aged 18 to 75 years. Subjects with known type 1 diabetes or clinical atherosclerotic CVD were excluded.

The Study of Inherited Risk of Coronary Atherosclerosis, Penn Diabetes Heart Study, and Philadelphia Area Metabolic Syndrome Network are all single-center studies that used the same general clinical research center, nursing staff, computed tomography scanner, and research laboratories. All study subjects were evaluated at the general clinical research center at Penn, as previously described.<sup>14</sup> CAC scores were determined by electron beam computed tomography (Imatron) according to the method of Agatston<sup>7</sup> from 40 continuous 3-mm thick computed tomograms. All Penn study protocols were approved by the Penn institutional review board, and all subjects provided written informed consent.

### **Statistical Analysis**

Because the distribution of CAC scores was positively skewed, the scores were natural log-transformed after adding 1. To evaluate the distribution of log-transformed CAC scores after regressing out the effects of age, sex, and age $\times$ sex, we obtained the residualized values of transformed CAC score in the AFCS sample. The residualized values were approximately normally distributed.

We followed a 2-stage approach in which we first identified a small number of promising SNPs in the Amish Discovery Set for follow-up, which we would then take forward for more rigorous statistical testing in the 2 independent replication cohorts. Our criteria for identifying SNPs of potential interest in the Amish discovery sample was that they be associated with CAC quantity at a liberal threshold of  $P < 0.01$ . To establish statistical significance, we then required that these SNPs be associated in both of the replication cohorts at probability values taking into account the number of SNPs tested in each cohort (ie, Bonferroni-corrected significance thresholds of 0.05/4=0.0125.) Finally, we combined evidence for association across all 3 studies in a metaanalysis, using a Bonferroni correction for 93 SNPs.

In the Amish Discovery sample, association analyses were carried out using a variance component approach suitable for analysis of large pedigrees. The approach used a mixed-effect model to assess the effect of genotype (included as a fixed effect) on CAC score while adjusting for covariates (age, sex, age $\times$ sex) and including a polygenic component (as a random effect) that accounted for the correlations in phenotype existing among related individuals. The polygenic component was modeled using the relationship matrix derived from the complete Amish pedigree structure. These analyses were carried out using Mixed Model Analysis for Pedigree software developed by 1 of the coauthors (J.R.O.). Pairwise linkage disequilibrium (LD) correlation statistics ( $r^2$ ) were computed for the Amish group using the Haploview software (<http://www.broad.mit.edu>).<sup>16</sup>

The SNPs that nominally associated with CAC score in the Amish sample were then tested for association in the GENOA and PennCAC samples. In GENOA, association analysis was carried out in R using linear mixed-effect models and included a random effect for family structure (ie, sibship). The effect of the *CYP24A1* SNPs on log-transformed (CAC score+1) was assessed with a random effect for sibship to account for the family structure (siblings) with adjustment for age, sex, and an age $\times$ sex interaction. The covariance structure was defined as compound symmetry. In PennCAC, the SNP association with log-transformed (CAC score+1) was tested using a linear regression model with *CYP24A1* genotype included as an independent variable and age, sex, and sex $\times$ age included as covariates. The analysis was carried out using the PLINK program, version 1.06.

For SNPs tested for association in all 3 cohorts, we combined association results across cohorts in a metaanalysis using a weighted fixed effects metaanalysis approach as implemented in the METAL program (<http://www.sph.umich.edu/csg/abecasis/Metal/index.html>).

### **Results**

The characteristics of participants in the Amish Discovery sample (n=697) and the GENOA (n=916) and PennCAC (n=2061) replication samples are summarized in Table 1. Mean ages of participants in the Amish and PennCAC samples were comparable (53 to 54 years) and slightly

**Table 1. Clinical Characteristics of the Study Populations**

|  | Discovery Sample<br>AFCS (n=697) | Replication Samples |                  |
|--|----------------------------------|---------------------|------------------|
|  |                                  | GENOA (n=916)       | PennCAC (n=2061) |
| Age, years   | 53.7 (12.8)                      | 58.1 (10.2)         | 53.4 (9.9)       |
| Male, n (%)  | 314 (45.1)                       | 376 (41.1)          | 726 (35.2)       |
| Body mass index, kg/m <sup>2</sup>                 | 28.2 (5.3)                       | 30.7 (6.3)          | 30.2 (5.7)       |
| Systolic blood pressure, mm Hg                     | 118.4 (16.4)                     | 130.8 (16.9)        | 128.5 (15.3)     |
| Diastolic blood pressure, mm Hg                    | 71.6 (8.8)                       | 74.4 (9.1)          | 76.9 (9.6)       |
| Total cholesterol, mg/dL                           | 208 (36)                         | 200.2 (33.4)        | 193.6 (40.9)     |
| Low-density lipoprotein cholesterol, mg/dL         | 132 (31)                         | 122.6 (31.9)        | 114.6 (34.9)     |
| High-density lipoprotein cholesterol, mg/dL        | 58 (15)                          | 52.6 (15.5)         | 49.0 (14.8)      |
| Triglycerides, median (interquartile range), mg/dL | 71 (52 to 105)                   | 135.5 (95 to 192)   | 124 (88 to 177)  |
| Hypertension, n (%)                                | 122 (17.5)                       | 643 (70.2)          | 902 (43.7)       |
| Diabetes, n (%)                                    | 17 (2.4)                         | 123 (13.4)          | 829 (40.2)       |
| Current smoker, n (%)                              | 55 (7.9)                         | 78 (8.2)            | 237 (11.5)       |
| Presence of CAC, n (%)                             | 312 (44.8)                       | 615 (67.1)          | 1364 (66.1)      |
| CAC score, median (interquartile range)            | 0 (0 to 66)                      | 13.2 (0 to 142)     | 11 (0 to 170)    |

For blood pressure measurements, some hypertension subjects were under blood pressure treatment. Values are mean (SD) unless otherwise indicated.

younger than the mean age in the GENOA sample (58 years). The proportion of men in the samples ranged from 35% to 45%, and the mean body mass index was 28.1 kg/m<sup>2</sup> in the Amish compared with 30.2 and 30.7 kg/m<sup>2</sup> in PennCAC and GENOA. By design, there was a substantially higher proportion of subjects with diabetes in the PennCAC sample (40.2%) compared with the Amish (2.4%) or the GENOA (13.4%) samples. By design, there was also a substantially higher proportion of subjects with hypertension in the GENOA sample (70.2%) compared with the Amish (17.5%) or PennCAC (43.7%) samples. In the GENOA sample, blood pressure measurements were recorded on blood pressure-lowering treatment.

Table 2 presents the sizes of the 3 genes, the numbers of SNPs genotyped, and summary results of the association testing. A total of 8 SNPs in *GC* (4 exons, 6822 bp), 3 SNPs in *CYP27B1* (9 exons, 4860 bp), 15 SNPs in *CYP24A1* (12

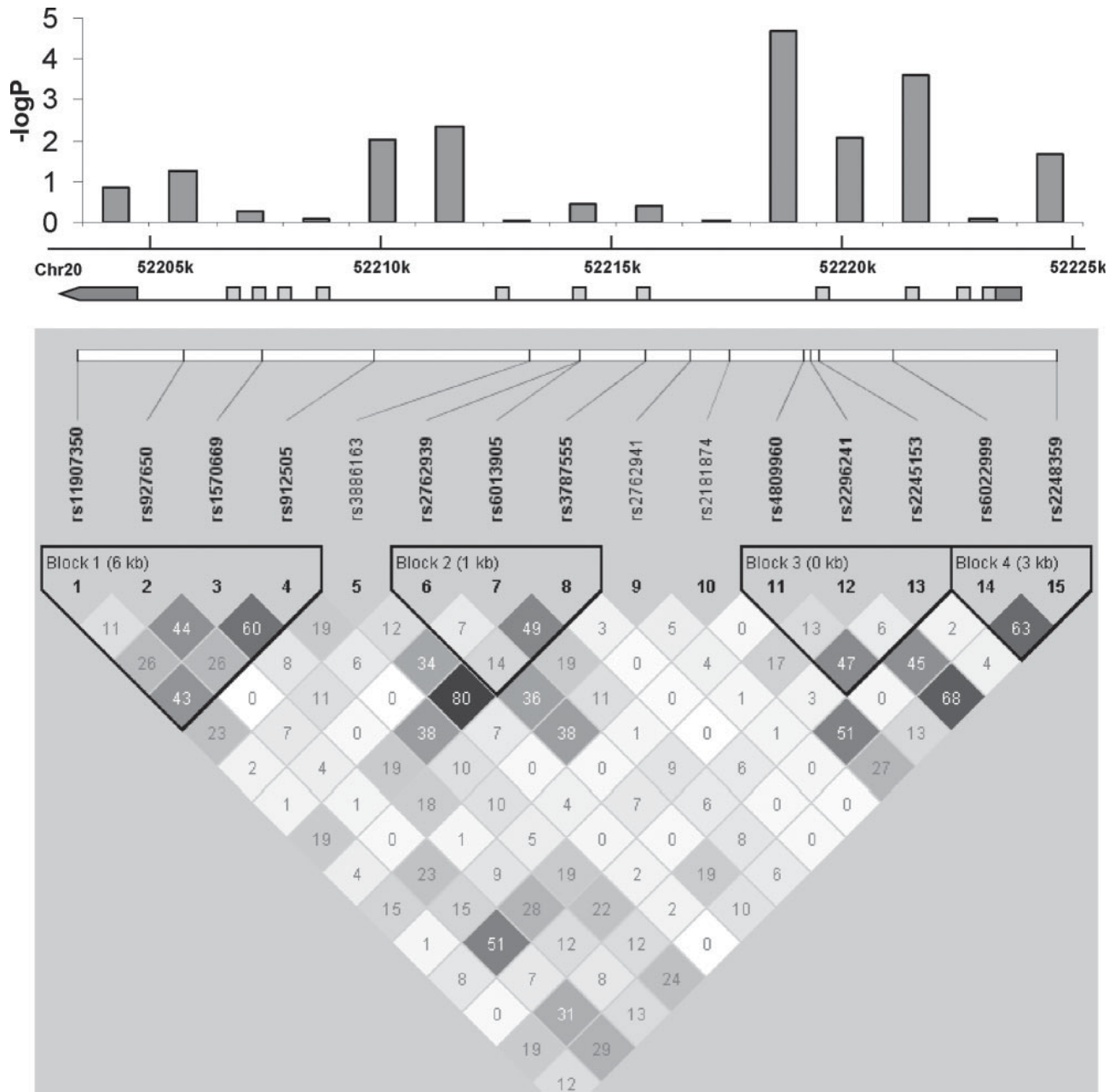
exons, 20 529 bp), and 67 SNPs in *VDR* (11 exons, 63 493 bp) were successfully genotyped and tested for association. We estimated the proportion of allelic variation in the genes captured by these SNPs in the HapMap CEU population. The 8 SNPs in *GC*, 3 SNPs in *CYP27B1*, and 67 SNPs in *VDR* (both IBC group 1 genes) captured 86%, 100%, and 89%, respectively, of all alleles having a frequency of 0.02 or higher at an  $r^2 > 0.80$ . The 15 SNPs in *CYP24A1* (a group 2 gene) captured 67% of all alleles having a frequency of 0.02 or higher at an  $r^2 > 0.80$ . There was no statistical evidence for deviation from Hardy-Weinberg equilibrium for any of the 93 successfully genotyped SNPs in the Amish based on a threshold of  $P < 0.0005$  (0.05/93). In the discovery sample, no SNPs in the *GC*, *CYP27B1*, or *VDR* gene were associated with CAC score at even a nominal probability value of 0.01 (see Supplemental Table I for individual SNP association results). In contrast, 4 of the 15 SNPs in *CYP24A1* were associated with CAC score, with nominal probability values ranging from 0.008 to 0.00003. The 4 SNPs in *CYP24A1* nominally associated with CAC quantity were 146 to 5155 bp apart from each other. SNP rs2762939 was located in 1 haplotype block and SNPs rs4809960, rs2296241, and rs2245153 in another block (all pairwise LD among rs4809960, rs2296241, and rs2245153:  $r^2 = 0.06$  to 0.47,  $D' = 0.99$ ). rs2762939 was not in LD with the other 3 SNPs (pairwise LD between rs2762939 and other 3 SNPs:  $r^2 < 0.07$ ,  $D' < 0.55$ ). The Figure shows the LD ( $r^2$ ) structure of the 15 SNPs in *CYP24A1* in the Amish sample and probability values for association of these SNPs with CAC score.

The 4 SNPs in *CYP24A1* with nominal probability value  $< 0.01$  in the Amish were then tested for association with CAC score in the GENOA and PennCAC populations. One of the 4 SNPs, rs2762939, was associated with CAC score in both the GENOA ( $P = 0.007$ ) and PennCAC ( $P = 0.01$ ) populations (Table 3). In all 3 populations, the C allele (minor allele frequency ranging from 0.25 to 0.31 across the 3

**Table 2. Description of 4 Vitamin D Metabolism Candidate Genes and Summary of Association Results With CAC in 3 Populations**

|  | <i>GC</i> | <i>CYP27B1</i> | <i>CYP24A1</i> | <i>VDR</i> |
|--|-----------|----------------|----------------|------------|
| Gene size,* bp                         | 6822      | 4860           | 20 529         | 63 493     |
| Exon count (coding exons count)        | 4 (4)     | 9 (9)          | 12 (11)        | 11 (8)     |
| No. of tagging SNPs (discovery sample) | 8         | 3              | 15             | 67         |
| No. of SNPs ( $P < 0.01$ )             |           |                |                |            |
| Discovery sample                       |           |                |                |            |
| AFCS                                   | 0         | 0              | 4              | 0          |
| Replication sample                     |           |                |                |            |
| GENOA                                  | ...       | ...            | 1              | ...        |
| UPENN                                  | ...       | ...            | 1              | ...        |

\*Only the translated region is shown.



**Figure.** The association between *CYP24A1* tagging SNPs and CAC score [Ln(CAC Score + 1), adjusted for age, sex, age×sex, and family structure] in the Amish. Pairwise LD ( $r^2$ ) values are shown. *CYP24A1* gene and exons are shown.

populations) was associated with lower CAC score. A formal test of heterogeneity indicated no significant differences in effect size across the 3 populations (Het  $P$  value=0.45). Combining the 3 probability values in a metaanalysis for the rs2762939–CAC score association across all 3 studies yielded a metaanalysis probability value of  $2.9 \times 10^{-6}$ . Combining results from the GENOA and PennCAC samples only (that is, excluding results from the Amish Discovery sample) yielded a probability value of  $2.8 \times 10^{-4}$ . There was no evidence for association of CAC score with any of the other 3 SNPs in the GENOA and PennCAC samples.

The relationship between rs2762939 and traditional risk factors was tested in AFCS. This SNP was not significantly associated with any of the blood pressure or serum lipid

phenotypes, nor was it associated with variation in serum 25(OH)D levels (data not shown). Consistent with these results, the association of rs2762939 with CAC quantity was essentially unchanged, with additional adjustment for low-density lipoprotein cholesterol and diabetes.

### Discussion

We have identified a common SNP, rs2762939, in *CYP24A1* that is associated with quantity of CAC. The gene product of *CYP24A1* is the major enzyme responsible for the catabolism of 1,25(OH)<sub>2</sub>D and 25(OH)D. Our data thus suggest that genetic variability in vitamin D homeostasis may contribute to the pathogenesis of CAC.

*CYP24A1* plays a pivotal role in maintaining vitamin D homeostasis. Deletion of *Cyp24a1* in mice causes

**Table 3. The Association Between *CYP24A1* SNPs and CAC Score\* in 3 Populations**

|           | Alleles† | AFCS (n=697) |              |                       | GENOA (n=916) |              |                      | PennCAC (n=2044) |              |          | Metaanalysis          |
|-----------|----------|--------------|--------------|-----------------------|---------------|--------------|----------------------|------------------|--------------|----------|-----------------------|
|           |          | Coded_F      | $\beta$ (SE) | <i>P</i>              | Coded_F       | $\beta$ (SE) | <i>P</i>             | Coded_F          | $\beta$ (SE) | <i>P</i> | <i>P</i>              |
| rs2762939 | C/G      | 0.25         | -0.36 (0.13) | $4.46 \times 10^{-3}$ | 0.31          | -0.36 (0.13) | $7.3 \times 10^{-3}$ | 0.26             | -0.18 (0.07) | 0.01     | $2.90 \times 10^{-6}$ |
| rs4809960 | C/T      | 0.11         | -0.75 (0.18) | $2.69 \times 10^{-5}$ | 0.22          | 0.17 (0.12)  | 0.15                 | 0.27             | -0.06 (0.07) | 0.39     | 0.04                  |
| rs2296241 | A/G      | 0.49         | -0.29 (0.11) | $7.67 \times 10^{-3}$ | 0.53          | 0.03 (0.10)  | 0.75                 | 0.56             | -0.05 (0.07) | 0.47     | 0.08                  |
| rs2245153 | C/T      | 0.06         | -0.87 (0.24) | $2.93 \times 10^{-4}$ | 0.18          | 0.17 (0.13)  | 0.21                 | 0.21             | -0.05 (0.08) | 0.54     | 0.09                  |

Coded\_F: frequency of coded allele.

\*Ln(CAC+1), adjusted for age, sex, age $\times$ sex, and family structure in AFCS and GENOA.

†Coded/noncoded allele.

1,25(OH)<sub>2</sub>D excess and hypercalcemia with severe bone mineralization defects and ectopic vascular calcification (renal calcium deposition) after chronic treatment of 1,25(OH)<sub>2</sub>D.<sup>17</sup> On the other hand, transgenic rats that constitutively express *Cyp24a1* develop atherosclerotic lesions in the aorta, which greatly progress with high-fat and high-cholesterol feeding.<sup>18</sup>

Although there is much circumstantial evidence, the role of vitamin D in cardiovascular health remains controversial.<sup>19</sup> There is some epidemiological evidence in humans supporting a role of vitamin D metabolites in the development of CAC. Watson et al measured serum 1,25(OH)<sub>2</sub>D in 153 asymptomatic individuals with high risk of coronary heart disease and 13 patients with familial hypercholesterolemia and reported, in both groups, an inverse correlation between serum 1,25(OH)<sub>2</sub>D and CAC.<sup>20</sup> Doherty et al reported that serum 1,25(OH)<sub>2</sub>D independently and inversely predicted CAC quantity in a sample of 283 asymptomatic subjects with risk factors for coronary heart disease.<sup>21</sup> Low 25(OH)D levels were also associated with subsequent development of CAC in the Multi-Ethnic Study of Atherosclerosis.<sup>22</sup> By contrast, there was no association between serum 25(OH)D levels and coronary calcification in a smaller study of 50 patients undergoing angiography.<sup>23</sup> In this Amish population, there is no correlation between season-adjusted 25(OH)D levels and prevalent CAC.<sup>24</sup> The discordant results observed could be due to limited power to detect associations of small magnitude, differences in patient characteristics among studies, or differences in assays used in these studies.

Although 25(OH)D is the best marker of vitamin D stores, 1,25(OH)<sub>2</sub>D is the active metabolite, and 25(OH)D levels might not reflect circulating 1,25(OH)<sub>2</sub>D levels. In addition, the unknown local 1,25(OH)<sub>2</sub>D production in the vasculature or in macrophages and other cell types involved in plaque formation might be more important than circulating levels for pathogenesis of vascular calcification.

This is the first demonstration of association between *CYP24A1* gene variants and vascular calcification in human population studies. *CYP24A1* SNPs or haplotypes have also been associated with other diseases in humans, including cancer<sup>25</sup> and asthma.<sup>26</sup> In their asthma study, Wjst et al reported that the *CYP24A1* haplotype that was moderately associated with asthma was also associated with 1,25(OH)<sub>2</sub>D, 25(OH)D, and IgE.<sup>26</sup> A recent genome-wide association analysis based on  $\approx 34$  000 individuals identified an association between rs6013897, located  $\approx 27.5$  kb centromeric of

*CYP24A1*, and 25(OH)D levels.<sup>27</sup> The SNP is 38.8 kb away from rs2762939, the SNP associated with CAC quantity in the present study, and there is little LD between rs6013897 and rs2762939 (HapMap CEU  $r^2=0.04$ ,  $D'=0.27$ ).

Despite the important role of *CYP24A1* in vitamin D metabolism, the mechanism linking rs2762939 to coronary calcification remains unclear. In neither the Amish study nor an independent sample of 546 premenopausal white women from Indiana (data not shown) was there any evidence for an association of rs2762939 with 25(OH)D levels. However, although an association of rs2762939 with 25(OH)D levels would have provided a clear potential mechanism linking this SNP to variation in CAC levels, there are several ways through which this SNP could affect CAC without influencing mean 25(OH)D levels. For example, this SNP or a SNP in high LD with this SNP could influence the conversion of active 1,25(OH)<sub>2</sub>D into its inactive metabolite without affecting 25(OH)D levels. One way to test this hypothesis would be to assess the association of rs2762939 with serum 1,25(OH)<sub>2</sub>D, but unfortunately we do not have these measurements in our study. Another potential mechanism is that this genetic variant may affect 24-hydroxylase expression and vitamin D metabolism locally in the endothelium, and these effects may not be correlated with circulating levels of vitamin D metabolites.

In summary, a common variant in the *CYP24A1* gene was associated with CAC quantity in 3 independent populations. This result suggests a role for vitamin D metabolism in coronary atherosclerosis. Future studies should elucidate the underlying mechanisms and signaling pathways that entwine vitamin D metabolism and vascular health. These studies may lead to novel screening and therapeutic options for the identification and treatment of individuals at increased risk for CVD events.

### Sources of Funding

The Amish component of this study was supported by National Institutes of Health Research Grants U01-HL72515 and R01-HL088119, F32AR059469, University of Maryland General Clinical Research Center Grant M01 RR 16500, an American Heart Association Scientist Development Grant (0830146N), and an American Heart Association Grant-In-Aid (0855400E). Partial funding was also provided by the Mid-Atlantic Nutrition and Obesity Research Center (P30 DK072488) and the Baltimore Department of Veterans Affairs Medical Center Geriatric Research and Education Clinical Center. GENOA was funded by National Institutes of Health Research Grants R01-HL087660, U10-HL54457 (Genetic Determinants of High BP in Three Racial Groups), and R01-HL091988 and

by a National Institutes of Health General Clinic Research Center Grant (M01-RR00585) awarded to Mayo Clinic Rochester. The PennCAC resource was supported by a Clinical and Translational Science Award (UL1RR024134) from the National Center for Research Resources and a Diabetes and Endocrine Research Center award (P20-DK 019525), both to the University of Pennsylvania. Dr Reilly is supported by R01-DK071224 from the National Institutes of Health. The study of Indiana Women was supported by National Institutes of Health Research Grants P01-AG-18397 and UL1-RR-025761.

## Disclosures

None.

## References

- Detrano R, Guerci AD, Carr JJ, Bild DE, Burke G, Folsom AR, Liu K, Shea S, Szklo M, Bluemke DA, O'Leary DH, Tracy R, Watson K, Wong ND, Kronmal RA. Coronary calcium as a predictor of coronary events in four racial or ethnic groups. *N Engl J Med*. 2008;358:1336–1345.
- Adams JS, Hewison M. Update in vitamin D. *J Clin Endocrinol Metab*. 2010;95:471–478.
- Jones G, Strugnelli SA, DeLuca HF. Current understanding of the molecular actions of vitamin D. *Physiol Rev*. 1998;78:1193–1231.
- Lehmann B, Meurer M. Vitamin D metabolism. *Dermatol Ther*. 2010;23:2–12.
- Tashiro K, Abe T, Oue N, Yasui W, Ryoji M. Characterization of vitamin D-mediated induction of the CYP 24 transcription. *Mol Cell Endocrinol*. 2004;226:27–32.
- Post W, Bielak LF, Ryan KA, Cheng YC, Shen H, Rumberger JA, Sheedy PF, Shuldiner AR, Peyser PA, Mitchell BD. Determinants of coronary artery and aortic calcification in the Old Order Amish. *Circulation*. 2007;115:717–724.
- Agatston AS, Janowitz WR, Hildner FJ, Zusmer NR, Viamonte M Jr, Detrano R. Quantification of coronary artery calcium using ultrafast computed tomography. *J Am Coll Cardiol*. 1990;15:827–832.
- Keating BJ, Tischfield S, Murray SS, Bhangale T, Price TS, Glessner JT, Galver L, Barrett JC, Grant SF, Farlow DN, Chandrupatla HR, Hansen M, Ajmal S, Papanicolaou GJ, Guo Y, Li M, Derohannessian S, de Bakker PI, Bailey SD, Montpetit A, Edmondson AC, Taylor K, Gai X, Wang SS, Fornage M, Shaikh T, Groop L, Boehnke M, Hall AS, Hattersley AT, Frackelton E, Patterson N, Chiang CW, Kim CE, Fabsitz RR, Uwehand W, Price AL, Munroe P, Caulfield M, Drake T, Boerwinkle E, Reich D, Whitehead AS, Cappola TP, Samani NJ, Lusk AJ, Schadt E, Wilson JG, Koenig W, McCarthy MI, Kathiresan S, Gabriel SB, Hakonarson H, Anand SS, Reilly M, Engert JC, Nickerson DA, Rader DJ, Hirschhorn JN, Fitzgerald GA. Concept, design and implementation of a cardiovascular gene-centric 50 k SNP array for large-scale genomic association studies. *PLoS One*. 2008;3:e3583.
- Shen H, Damcott CM, Rampersaud E, Pollin TI, Horenstein RB, McArdle PF, Peyser PA, Bielak LF, Post WS, Chang YP, Ryan KA, Miller M, Rumberger JA, Sheedy PF II, Shelton J, O'Connell JR, Shuldiner AR, Mitchell BD. Familial defective ApoB-100 is a major cause of increased LDL-cholesterol and coronary artery calcification in the Old Order Amish. *Arch Intern Med*. In press.
- FBPP Investigators. Multi-center genetic study of hypertension: the Family Blood Pressure Program (FBPP). *Hypertension*. 2002;39:3–9.
- Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL Jr, Jones DW, Materson BJ, Oparil S, Wright JT Jr, Roccella EJ, Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure, National Heart, Lung, and Blood Institute, National High Blood Pressure Education Program Coordinating Committee. Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Hypertension*. 2003;42:1206–1252.
- Peyser PA, Bielak LF, Chu JS, Turner ST, Ellsworth DL, Boerwinkle E, Sheedy PF II. Heritability of coronary artery calcium quantity measured by electron beam computed tomography in asymptomatic adults. *Circulation*. 2002;106:304–308.
- Brown RJ, Edmondson AC, Griffon N, Hill TB, Fuki IV, Badellino KO, Li M, Wolfe ML, Reilly MP, Rader DJ. A naturally occurring variant of endothelial lipase associated with elevated HDL exhibits impaired synthesis. *J Lipid Res*. 2009;50:1910–1916.
- Reilly MP, Wolfe ML, Localio AR, Rader DJ. Study of Inherited Risk of Coronary Atherosclerosis. C-reactive protein and coronary artery calcification: the Study of Inherited Risk of Coronary Atherosclerosis (SIRCA). *Arterioscler Thromb Vasc Biol*. 2003;23:1851–1856.
- Bagheri R, Schutta M, Cumarantunge RG, Wolfe ML, Terembula K, Hoffman B, Schwartz S, Kimmel SE, Farouk S, Iqbal N, Reilly MP. Value of electrocardiographic and ankle-brachial index abnormalities for prediction of coronary atherosclerosis in asymptomatic subjects with type 2 diabetes mellitus. *Am J Cardiol*. 2007;99:951–955.
- Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics*. 2005;21:263–265.
- St-Arnaud R, Arabian A, Travers R, Barletta F, Raval-Pandya M, Chapin K, Depovere J, Mathieu C, Christakos S, Demay MB, Glorieux FH. Deficient mineralization of intramembranous bone in vitamin D-24-hydroxylase-ablated mice is due to elevated 1,25-dihydroxyvitamin D and not to the absence of 24,25-dihydroxyvitamin D. *Endocrinology*. 2000;141:2658–2666.
- Kasuga H, Hosogane N, Matsuoka K, Mori I, Sakura Y, Shimakawa K, Shinki T, Suda T, Taketomi S. Characterization of transgenic rats constitutively expressing vitamin D-24-hydroxylase gene. *Biochem Biophys Res Commun*. 2002;297:1332–1338.
- Swales HH, Wang TJ. Vitamin D and cardiovascular disease risk: emerging evidence. *Curr Opin Cardiol*. 2010;22:513–517.
- Watson KE, Abrolat ML, Malone LL, Hoeg JM, Doherty T, Detrano R, Demer LL. Active serum vitamin D levels are inversely correlated with coronary calcification. *Circulation*. 1997;96:1755–1760.
- Doherty TM, Tang W, Dascalos S, Watson KE, Demer LL, Shavelle RM, Detrano RC. Ethnic origin and serum levels of 1 $\alpha$ ,25-dihydroxyvitamin D3 are independent predictors of coronary calcium mass measured by electron-beam computed tomography. *Circulation*. 1997;96:1477–1481.
- de Boer IH, Kestenbaum B, Shoben AB, Michos ED, Sarnak MJ, Siscovick DS. 25-hydroxyvitamin D levels inversely associate with risk for developing coronary artery calcification. *J Am Soc Nephrol*. 2009;20:1805–1812.
- Arad Y, Spadaro LA, Roth M, Scordo J, Goodman K, Sherman S, Lerner G, Newstein D, Guerci AD. Serum concentration of calcium, 1,25 vitamin D and parathyroid hormone are not correlated with coronary calcifications: an electron beam computed tomography study. *Coron Artery Dis*. 1998;9:513–518.
- Michos ED, Streeten EA, Ryan KA, Rampersaud E, Peyser PA, Bielak LF, Shuldiner AR, Mitchell BD, Post W. Serum 25-hydroxyvitamin D levels are not associated with subclinical vascular disease or C-reactive protein in the old order Amish. *Calcif Tissue Int*. 2009;84:195–202.
- McCullough ML, Bostick RM, Mayo TL. Vitamin D gene pathway polymorphisms and risk of colorectal, breast, and prostate cancer. *Annu Rev Nutr*. 2009;29:111–132.
- Wjst M, Altmüller J, Faus-Kessler T, Braig C, Bahnweg M, Andre E. Asthma families show transmission disequilibrium of gene variants in the vitamin D metabolism and signalling pathway. *Respir Res*. 2006;7:60.
- Wang TJ, Zhang F, Richards JB, Kestenbaum B, van Meurs JB, Berry D, Kiel DP, Streeten EA, Ohlsson C, Koller DL, Peltonen L, Cooper JD, O'Reilly PF, Houston DK, Glazer NL, Vandenput L, Peacock M, Shi J, Rivadeneira F, McCarthy MI, Anneli P, de Boer IH, Mangino M, Kato B, Smyth DJ, Booth SL, Jacques PF, Burke GL, Goodarzi M, Cheung CL, Wolf M, Rice K, Goltzman D, Hidioglou N, Ladouceur M, Wareham NJ, Hocking LJ, Hart D, Arden NK, Cooper C, Malik S, Fraser WD, Hartikainen AL, Zhai G, Macdonald HM, Forouhi NG, Loos RJ, Reid DM, Hakim A, Dennison E, Liu Y, Power C, Stevens HE, Jaana L, Vasani RS, Soranzo N, Bojunga J, Psaty BM, Lorentzen M, Forouf T, Harris TB, Hoffman A, Jansson JO, Cauley JA, Uitterlinden AG, Gibson Q, Jarvelin MR, Karasik D, Siscovick DS, Econs MJ, Kritchevsky SB, Florez JC, Todd JA, Dupuis J, Hyppönen E, Spector TD. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. *Lancet*. 2010;376:180–188.

**Supplemental Table 1. Association of tagging SNPs in CYP24A1, CYP27B1, VDR, and GC with coronary artery calcification score**

| SNP        | CHR | POS      | Function       | NON_ALLELE | ALLELE | EFFECT  | EFFECT_ALLELE | BETA     | SNP      | SE_SNP  | P | GENE |
|------------|-----|----------|----------------|------------|--------|---------|---------------|----------|----------|---------|---|------|
| rs11907350 | 20  | 52203846 | untranslated-3 | A          | G      | 0.91822 | -0.33329      | 0.204145 | 1.03E-01 | CYP24A1 |   |      |
| rs927650   | 20  | 52206148 | intron         | A          | G      | 0.42539 | -0.195428     | 0.112328 | 8.23E-02 | CYP24A1 |   |      |
| rs1570669  | 20  | 52207834 | intron         | A          | G      | 0.25251 | -0.049094     | 0.132683 | 7.11E-01 | CYP24A1 |   |      |
| rs912505   | 20  | 52210248 | intron         | A          | G      | 0.17001 | 0.004953      | 0.152883 | 9.74E-01 | CYP24A1 |   |      |
| rs3886163  | 20  | 52213600 | intron         | A          | G      | 0.73386 | -0.324864     | 0.126973 | 1.07E-02 | CYP24A1 |   |      |
| rs2762939  | 20  | 52214658 | intron         | C          | G      | 0.25144 | -0.360752     | 0.126432 | 4.46E-03 | CYP24A1 |   |      |
| rs6013905  | 20  | 52214671 | intron         | A          | G      | 0.17862 | 0.0047        | 0.141657 | 9.74E-01 | CYP24A1 |   |      |
| rs3787555  | 20  | 52216098 | intron         | A          | C      | 0.68938 | -0.118733     | 0.123326 | 3.36E-01 | CYP24A1 |   |      |
| rs2762941  | 20  | 52217059 | intron         | A          | G      | 0.62912 | 0.118748      | 0.111746 | 2.88E-01 | CYP24A1 |   |      |
| rs2181874  | 20  | 52217885 | intron         | A          | G      | 0.65352 | 0.001754      | 0.118783 | 9.88E-01 | CYP24A1 |   |      |
| rs4809960  | 20  | 52219480 | intron         | A          | G      | 0.1076  | -0.748799     | 0.177162 | 2.69E-05 | CYP24A1 |   |      |
| rs2296241  | 20  | 52219626 | coding-synon   | A          | G      | 0.50861 | 0.291531      | 0.109014 | 7.67E-03 | CYP24A1 |   |      |
| rs2245153  | 20  | 52219813 | intron         | A          | G      | 0.05667 | -0.873218     | 0.239911 | 2.93E-04 | CYP24A1 |   |      |
| rs6022999  | 20  | 52221420 | intron         | A          | G      | 0.3056  | -0.026145     | 0.119543 | 8.27E-01 | CYP24A1 |   |      |
| rs2248359  | 20  | 52224925 | near-gene-3    | A          | G      | 0.59469 | 0.258176      | 0.109729 | 1.89E-02 | CYP24A1 |   |      |
| rs4646537  | 12  | 56443548 | intron         | A          | C      | 0.01076 | -0.272948     | 0.529236 | 6.06E-01 | CYP27B1 |   |      |
| rs4646536  | 12  | 56444255 | intron         | A          | G      | 0.3967  | -0.122191     | 0.112671 | 2.79E-01 | CYP27B1 |   |      |
| rs8176345  | 12  | 56444825 | coding-synon   | A          | G      | 0.9627  | 0.328565      | 0.279507 | 2.40E-01 | CYP27B1 |   |      |
| rs2544043  | 12  | 46522817 | untranslated-3 | C          | G      | 0.86416 | -0.250254     | 0.159971 | 1.18E-01 | VDR     |   |      |
| rs9729     | 12  | 46522890 | untranslated-3 | A          | C      | 0.41894 | 0.112472      | 0.11488  | 3.28E-01 | VDR     |   |      |
| rs11574121 | 12  | 46524195 | untranslated-3 | A          | G      | 0.96341 | 0.132623      | 0.301421 | 6.60E-01 | VDR     |   |      |
| rs3858733  | 12  | 46524234 | untranslated-3 | A          | C      | 0.03587 | 0.527108      | 0.294822 | 7.42E-02 | VDR     |   |      |
| rs3847987  | 12  | 46524335 | untranslated-3 | A          | C      | 0.68149 | 0.041957      | 0.116882 | 7.20E-01 | VDR     |   |      |
| rs11574119 | 12  | 46524359 | untranslated-3 | C          | G      | 0.9648  | -0.531379     | 0.296891 | 7.39E-02 | VDR     |   |      |
| rs731236   | 12  | 46525024 | coding-synon   | A          | G      | 0.25897 | -0.081723     | 0.126436 | 5.18E-01 | VDR     |   |      |
| rs11574113 | 12  | 46525167 | intron         | C          | G      | 0.69584 | 0.112505      | 0.118282 | 3.42E-01 | VDR     |   |      |
| rs1544410  | 12  | 46526102 | intron         | A          | G      | 0.73745 | 0.086007      | 0.125669 | 4.94E-01 | VDR     |   |      |
| rs2525044  | 12  | 46528523 | intron         | A          | G      | 0.62195 | -0.025093     | 0.114655 | 8.27E-01 | VDR     |   |      |
| rs7962898  | 12  | 46529104 | intron         | A          | G      | 0.41392 | 0.103285      | 0.114449 | 3.67E-01 | VDR     |   |      |
| rs7963776  | 12  | 46529644 | intron         | A          | G      | 0.41451 | 0.097389      | 0.114381 | 3.95E-01 | VDR     |   |      |
| rs2239185  | 12  | 46530826 | intron         | A          | G      | 0.41392 | 0.103285      | 0.114449 | 3.67E-01 | VDR     |   |      |
| rs7975128  | 12  | 46532095 | intron         | A          | G      | 0.73745 | 0.086007      | 0.125669 | 4.94E-01 | VDR     |   |      |
| rs11574099 | 12  | 46534566 | intron         | A          | G      | 0.96413 | -0.527108     | 0.294822 | 7.42E-02 | VDR     |   |      |
| rs7305032  | 12  | 46536127 | intron         | A          | G      | 0.37805 | 0.025093      | 0.114655 | 8.27E-01 | VDR     |   |      |
| rs11574085 | 12  | 46537061 | intron         | A          | T      | 0.01506 | 0.278413      | 0.440083 | 5.27E-01 | VDR     |   |      |
| rs11168266 | 12  | 46537800 | intron         | A          | G      | 0.40029 | 0.101838      | 0.113146 | 3.68E-01 | VDR     |   |      |
| rs11168267 | 12  | 46537809 | intron         | A          | G      | 0.70231 | 0.069172      | 0.118963 | 5.61E-01 | VDR     |   |      |
| rs11168268 | 12  | 46538079 | intron         | A          | G      | 0.39168 | 0.050666      | 0.1132   | 6.55E-01 | VDR     |   |      |
| rs2238140  | 12  | 46538931 | intron         | A          | G      | 0.43185 | 0.128082      | 0.112734 | 2.56E-01 | VDR     |   |      |

|            |    |          |          |   |   |         |           |          |          |     |
|------------|----|----------|----------|---|---|---------|-----------|----------|----------|-----|
| rs11574077 | 12 | 46539194 | intron   | A | G | 0.02511 | -0.450632 | 0.361414 | 2.13E-01 | VDR |
| rs2248098  | 12 | 46539623 | intron   | A | G | 0.56815 | -0.128082 | 0.112734 | 2.56E-01 | VDR |
| rs2239182  | 12 | 46541678 | intron   | A | G | 0.29412 | 0.013803  | 0.120981 | 9.09E-01 | VDR |
| rs2107301  | 12 | 46541837 | intron   | A | G | 0.58824 | 0.100536  | 0.107989 | 3.52E-01 | VDR |
| rs2283342  | 12 | 46542126 | intron   | A | G | 0.08321 | -0.046635 | 0.197783 | 8.14E-01 | VDR |
| rs2239181  | 12 | 46542216 | intron   | A | C | 0.20761 | 0.064978  | 0.135043 | 6.31E-01 | VDR |
| rs1540339  | 12 | 46543593 | intron   | A | G | 0.53874 | 0.083287  | 0.106859 | 4.36E-01 | VDR |
| rs2239179  | 12 | 46544033 | intron   | A | G | 0.26112 | -0.030288 | 0.126626 | 8.11E-01 | VDR |
| rs12717991 | 12 | 46545393 | intron   | A | G | 0.53874 | 0.083287  | 0.106859 | 4.36E-01 | VDR |
| rs12721370 | 12 | 46548340 | intron   | A | C | 0.79842 | -0.040697 | 0.137056 | 7.67E-01 | VDR |
| rs886441   | 12 | 46549231 | intron   | A | G | 0.27762 | 0.131741  | 0.119814 | 2.72E-01 | VDR |
| rs2189480  | 12 | 46550095 | intron   | A | C | 0.72023 | -0.014183 | 0.121721 | 9.07E-01 | VDR |
| rs3782905  | 12 | 46552434 | intron   | C | G | 0.76686 | 0.057827  | 0.132341 | 6.62E-01 | VDR |
| rs2239186  | 12 | 46555677 | intron   | A | G | 0.12626 | -0.02533  | 0.165005 | 8.78E-01 | VDR |
| rs11574052 | 12 | 46558372 | intron   | A | G | 0.9175  | 0.453433  | 0.195203 | 2.05E-02 | VDR |
| rs11168275 | 12 | 46558542 | intron   | A | G | 0.25466 | -0.082225 | 0.12225  | 5.01E-01 | VDR |
| rs10783218 | 12 | 46559010 | intron   | A | G | 0.97561 | -0.219364 | 0.355311 | 5.37E-01 | VDR |
| rs10735810 | 12 | 46559162 | missense | A | G | 0.76901 | -0.158203 | 0.129567 | 2.22E-01 | VDR |
| rs2254210  | 12 | 46559981 | intron   | A | G | 0.63343 | 0.031186  | 0.111376 | 7.80E-01 | VDR |
| rs11574044 | 12 | 46562101 | intron   | A | C | 0.12482 | -0.09267  | 0.173503 | 5.93E-01 | VDR |
| rs11574042 | 12 | 46562446 | intron   | C | G | 0.96055 | -0.335328 | 0.25921  | 1.96E-01 | VDR |
| rs11574040 | 12 | 46562699 | intron   | A | G | 0.18077 | -0.286106 | 0.136691 | 3.67E-02 | VDR |
| rs2238136  | 12 | 46563980 | intron   | A | G | 0.78838 | -0.224657 | 0.129552 | 8.33E-02 | VDR |
| rs1989969  | 12 | 46564277 | intron   | A | G | 0.5165  | 0.038915  | 0.109241 | 7.22E-01 | VDR |
| rs2238135  | 12 | 46564457 | intron   | C | G | 0.79699 | -0.163996 | 0.132321 | 2.16E-01 | VDR |
| rs2853564  | 12 | 46564754 | intron   | A | G | 0.4835  | -0.038915 | 0.109241 | 7.22E-01 | VDR |
| rs4760648  | 12 | 46566932 | intron   | A | G | 0.65208 | 0.040225  | 0.117033 | 7.31E-01 | VDR |
| rs2853559  | 12 | 46569072 | intron   | A | G | 0.51148 | 0.017199  | 0.1089   | 8.75E-01 | VDR |
| rs11168287 | 12 | 46571681 | intron   | A | G | 0.46695 | 0.042899  | 0.107308 | 6.89E-01 | VDR |
| rs4328262  | 12 | 46571915 | intron   | A | C | 0.434   | 0.05134   | 0.108349 | 6.36E-01 | VDR |
| rs4334089  | 12 | 46572282 | intron   | A | G | 0.77618 | 0.127245  | 0.129928 | 3.28E-01 | VDR |
| rs4237855  | 12 | 46573470 | intron   | A | G | 0.39024 | 0.03819   | 0.111397 | 7.32E-01 | VDR |
| rs11574026 | 12 | 46574513 | intron   | A | G | 0.9627  | -0.099396 | 0.274019 | 7.17E-01 | VDR |
| rs3890733  | 12 | 46575640 | intron   | A | G | 0.63415 | -0.085554 | 0.113059 | 4.49E-01 | VDR |
| rs12427078 | 12 | 46578658 | intron   | A | G | 0.02367 | 0.420214  | 0.359906 | 2.43E-01 | VDR |
| rs10875695 | 12 | 46579304 | intron   | A | C | 0.78264 | 0.166778  | 0.129031 | 1.97E-01 | VDR |
| rs7136534  | 12 | 46580893 | intron   | A | G | 0.78264 | 0.166778  | 0.129031 | 1.97E-01 | VDR |
| rs12721377 | 12 | 46581618 | intron   | A | G | 0.02654 | 0.041694  | 0.351176 | 9.06E-01 | VDR |
| rs10783219 | 12 | 46581755 | intron   | A | T | 0.39024 | 0.03819   | 0.111397 | 7.32E-01 | VDR |
| rs11574024 | 12 | 46582488 | intron   | A | C | 0.967   | -0.197643 | 0.306549 | 5.19E-01 | VDR |
| rs7299460  | 12 | 46582535 | intron   | A | G | 0.7561  | 0.14837   | 0.123888 | 2.31E-01 | VDR |
| rs4760658  | 12 | 46582753 | intron   | A | G | 0.36585 | 0.085554  | 0.113059 | 4.49E-01 | VDR |

|            |    |          |             |   |   |         |           |          |          |             |
|------------|----|----------|-------------|---|---|---------|-----------|----------|----------|-------------|
| rs11574005 | 12 | 46585837 | near-gene-3 | A | G | 0.97346 | -0.041694 | 0.351176 | 9.06E-01 | VDR         |
| rs7139166  | 12 | 46586601 | near-gene-3 | C | G | 0.44118 | 0.019754  | 0.108593 | 8.56E-01 | VDR         |
| rs11568820 | 12 | 46588812 | unknown     | A | G | 0.82138 | 0.076691  | 0.139116 | 5.82E-01 | VDR         |
| rs7970314  | 12 | 46594441 | unknown     | A | G | 0.17862 | -0.076691 | 0.139116 | 5.82E-01 | EAR/VDR/VDR |
| rs454715   | 19 | 53806048 | near-gene-5 | A | C | 0.67575 | -0.2005   | 0.115476 | 8.30E-02 | GC          |
| rs2617800  | 19 | 53807946 | near-gene-5 | A | C | 0.97776 | 0.280935  | 0.379046 | 4.59E-01 | GC          |
| rs447802   | 19 | 53808171 | near-gene-5 | A | G | 0.39727 | -0.218443 | 0.108438 | 4.43E-02 | GC          |
| rs2292342  | 19 | 53810183 | near-gene-5 | A | G | 0.94524 | 0.094199  | 0.237409 | 6.92E-01 | GC          |
| rs369880   | 19 | 53812246 | intron      | A | G | 0.82999 | 0.124154  | 0.142021 | 3.82E-01 | GC          |
| rs2303147  | 19 | 53834837 | intron      | A | G | 0.82999 | 0.124154  | 0.142021 | 3.82E-01 | GC          |
| rs11880333 | 19 | 53836602 | intron      | A | G | 0.94476 | 0.084492  | 0.236473 | 7.21E-01 | GC          |
| rs8103121  | 19 | 53849950 | intron      | A | G | 0.29699 | 0.03514   | 0.115398 | 7.61E-01 | GC          |

The SNP position is on NCBI build 36 map