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Common variants in the calcium-sensing receptor gene are associated with total serum calcium levels

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Serum calcium levels are tightly regulated. We performed genome-wide association studies (GWAS) in population-based studies participating in the CHARGE Consortium to uncover common genetic variations associated with total serum calcium levels. GWAS of serum calcium concentrations was performed in 20 611 individuals of European ancestry for ~2.5 million genotyped and imputed single-nucleotide polymorphisms (SNPs). The SNP with the lowest *P*-value was rs17251221 ($P = 2.4 * 10^{-22}$, minor allele frequency 14%) in the calcium-sensing receptor gene (*CASR*). This lead SNP was associated with higher serum calcium levels [0.06 mg/dl (0.015 mmol/l) per copy of the minor G allele] and accounted for 0.54% of the variance in serum calcium concentrations. The identification of variation in *CASR* that influences serum calcium concentration confirms the results of earlier candidate gene studies. The G allele of rs17251221 was also associated with higher serum magnesium levels ($P = 1.2 * 10^{-3}$), lower serum phosphate levels ($P = 2.8 * 10^{-7}$) and lower

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bone mineral density at the lumbar spine ($P = 0.038$), but not the femoral neck. No additional genomic loci contained SNPs associated at genome-wide significance ($P < 5 * 10^{-8}$). These associations resemble clinical characteristics of patients with familial hypocalciuric hypercalcemia, an autosomal-dominant disease arising from rare inactivating mutations in the *CASR* gene. We conclude that common genetic variation in the *CASR* gene is associated with similar but milder features in the general population.

INTRODUCTION

Calcium homeostasis is a vital process for the maintenance of healthy teeth and bones, cell signaling, coagulation, muscle contraction and multiple other functions. In health, levels of circulating ionized calcium are tightly maintained in a narrow range between 4.5 and 5.6 mg/dl (1.1–1.4 mmol/l) by a homeostatic network known as the 'calcistat' (1). The biochemical and endocrine components of this regulatory system are well known, having largely been deduced from the study of disease states characterized by abnormalities in serum calcium concentration. Parathyroid hormone (PTH) is the main regulator of minute-to-minute calcium balance and a key regulator of its release is the calcium-sensing receptor (CASR), located mainly in the plasma membrane of chief cells of the parathyroid gland and in cells of the renal tubule (2).

Less is known about the constitutive or genetic factors that determine serum calcium concentration in the general population, although twin studies suggest the heritability for total calcium to be between 33 and 78% (3–5). The common A986S polymorphism of the *CASR* gene was previously shown to have a significant effect on extracellular calcium in a small study of healthy women (6). However, the majority of genetic contribution to common variation in serum calcium concentration remains to be established.

Thus, our aim was to identify genetic loci associated with serum calcium concentration by conducting genome-wide association studies (GWAS) in six populations (20 611 participants of European ancestry) from the CHARGE Consortium (7).

RESULTS

Study sample characteristics are shown in Table 1; overall, 20 611 participants were available for genome-wide association analysis.

In the family-based Framingham Heart Study (FHS), serum calcium concentrations were found to be heritable at 39.6% ($P < 0.001$). The quantile–quantile plot of observed versus expected $-\log_{10}$(*P*-values) demonstrated an excess of small *P*-values (Supplementary Material, Fig. S1A). Supplementary Material, Figure S1B displays $-\log_{10}$(*P*-values) for the association between each SNP and serum calcium levels by genomic position; a clear genome-wide significant association signal is evident on chromosome 3. Even after exclusion of this region, an excess of small *P*-values was observed (inset of Supplementary Material, Fig. S1A), suggesting the presence of additional genetic variants influencing serum calcium levels below the genome-wide significance threshold.

The SNP with the lowest *P*-value, rs17251221, is located in an intron of the *CASR* gene on chromosome 3; the meta-analysis *P*-value was $2.4 * 10^{-22}$. Study-specific *P*-values for the association between serum calcium and rs17251221 are shown in Table 2. Mean serum calcium levels were 0.06 mg/dl (0.015 mmol/l) higher per copy of the minor G allele. The SNP explained 0.54% of the variance of circulating serum calcium levels. Estimates for a nearby coding SNP in high linkage disequilibrium (LD) [rs1801725 (A986S), $r^2 = 0.93$ in HapMap CEU] are also provided (Table 2 legend). Figure 1 depicts SNP associations with serum calcium levels in this genomic region. Although the minor G allele of rs17251221 (minor allele frequency of 14%) was associated with higher serum calcium levels, there were also SNPs in the *CASR* gene for which the minor allele of ~16% frequency associated with lower serum calcium levels and that showed low pair-wise correlation with rs17251221 ($r^2 < 0.04$): the lowest *P*-value was observed for rs9817571 ($P = 2.9 * 10^{-6}$).

Two sets of sensitivity analyses correcting the serum calcium concentration for albumin concentrations showed essentially unchanged multivariable-adjusted associations between serum calcium and rs17251221 (effect estimate for total calcium: −0.017 per G allele, $P = 4.6 * 10^{-14}$; effect estimate for corrected calcium based on study-specific albumin values: −0.017 per G allele, $P = 5.2 * 10^{-16}$; effect estimate for total calcium corrected for standard albumin: −0.017 per G allele, $P = 4.1 * 10^{-14}$; for the description of analyses, see Materials and Methods). Further sensitivity analyses were conducted to address a possible effect introduced by the presence of study participants with chronic kidney disease (CKD), defined as estimated glomerular filtration rate (eGFR) <60 ml/min/1.73 m^2.The correlation of the SNP effect estimates from genome-wide analyses before and after excluding individuals with CKD was 0.98. Effect estimates for association between each copy of the G allele of rs17251221 and serum calcium and serum phosphate were very similar before and after the exclusion of individuals with CKD, indicating that the presence of individuals with CKD in our data set is unlikely to influence the association between rs17251221 and total calcium concentrations.

Rare loss-of-function mutations in the *CASR* can cause autosomal-dominant familial hypocalciuric hypercalcemia (FHH), which presents with hypercalcemia, hypocalciuria, relatively high levels of PTH for the degree of hypercalcemia and also mild hypermagnesemia. In addition, an association between CASR haplotypes and primary hyperparathyroidism has also been described (8). We therefore additionally examined the association between rs17251221 and serum magnesium and phosphate levels as well as PTH (see Materials and Methods for details). The G allele of rs17251221 was also associated with higher serum magnesium levels ($P = 1.2 * 10^{-3}$, lower serum phosphate levels ($P = 2.8 * 10^{-7}$), but not with PTH levels ($P = 0.12$), although the direction of the observed PTH

Table 1. Characteristics of participants to the studies

	FHS	ARIC	CHS	Rotterdam	HABC	AGES
Participants	2853	9049	1770	3436	1554	1949
Calcium (mg/dl)[a]	9.61 (0.37)	9.77 (0.41)	9.45 (0.35)	9.54 (0.63)	8.82 (0.42)	9.42 (0.48)
Female (%)	52.3 (1491)	52.9 (4790)	69.4 (1228)	62.5 (2149)	46.8 (728)	56.6 (1103)
Age (years)	43.6 (9.8)	54.3 (5.7)	71.2 (4.6)	70.3 (8.6)	74.8 (2.8)	77.0 (5.4)
CKD[b] (%)	4.0 (113)	2.9 (258)	16.6 (292)	13.5 (463)	24.8 (386)	20.8 (405)
PTH above reference limit[c] (%)	NA	NA	23.0 (402)	NA	NA	NA

Data are presented as mean (SD) for continuous traits and percent (*n*) for dichotomous traits. HABC, Health ABC Study.
[a]To convert to SI units (mmol/l), multiply by 0.25.
[b]CKD defined as eGFR < 60 ml/min/1.73 m^2.
[c]PTH upper reference limit 65 pg/ml.

association was consistent with that seen in FHH. The associations between serum calcium and rs17251221 were robust following the additional inclusion of serum phosphate concentrations as a covariate.

Previous reports on common variation in the *CASR* gene provided conflicting results on the association with bone mineral density (BMD) (9–12). We therefore evaluated the association between rs17251221 with lumbar spine and femoral neck BMD in the large Genetic Factors for Osteoporosis (GEFOS) Consortium (13): each copy of the G allele was associated with 0.04 standard deviations lower lumbar spine BMD ($P = 0.038$) but not with femoral neck BMD ($P = 0.4$).

A rare gain-of-function variant of the CASR, the R990G variant encoded by rs1042636, has been reported to associate with hypercalciuria and kidney stone disease (8,14). We therefore evaluated the association between this SNP and kidney stone disease as outlined in Materials and Methods. Consistent with previous reports, the SNP showed a significant association with kidney stone disease at a type I error probability of 0.05 (OR = 1.15 per minor G allele, 95% confidence interval 0.76–0.99, $P = 0.036$). Per each additional copy of the G allele (encoding for the 990G variant), serum calcium levels were lower (effect size -0.026, $P = 9 * 10^{-4}$).

Additional SNPs associated with serum calcium levels at $P < 10^{-5}$ from our meta-analysis are presented in Supplementary Material, Table S2. Notably, variants in the *GCKR* gene, which have shown multiple pleiotropic associations with glycemic traits, kidney function and serum lipid, urate and CRP levels (http://www.genome.gov/GWAstudies/), were associated with serum calcium at $P < 1 * 10^{-5}$. Neither rs17251221 nor rs1801725 were associated with gene expression among publicly available eQTL data sets, which have been described previously (15). In addition, there were no other variants in the *CASR* gene correlated with our lead SNP at $r^2 > 0.5$ that showed an association with CASR transcript levels.

DISCUSSION

We have identified common variants in the CASR gene in a large meta-analysis of GWAS of serum calcium levels, including data from 20 611 individuals of European ancestry. Each copy of the minor G allele at rs17251221, which is highly correlated with the *CASR* A986S polymorphism, was associated with higher serum calcium levels, higher serum magnesium levels, lower serum phosphate levels and lower lumbar spine, but not femoral neck, BMD in our population-based study.

Previous studies of genetic determinants of serum calcium levels have mostly focussed on the study of rare Mendelian disorders featuring abnormal serum calcium levels (16). The common *CASR* A986S polymorphism was associated with corrected serum calcium levels in a previous candidate gene study (6). Our genome-wide approach confirms this earlier findings which is of interest as results from early candidate gene studies have typically proven difficult to replicate (17). Results from a second GWAS of serum calcium levels also support the identification of common variation in the *CASR* gene as an important determinant of serum calcium levels (18). Our study extends the literature in this area by characterizing associations between an identified calcium-increasing variant and other physiologically related phenotypes, including serum magnesium and phosphate levels, as well as BMD. The associations we observe resemble features of a known Mendelian disorder of calcium metabolism, FHH. Further, we corroborate the heritability of serum calcium levels, previously estimated from twin studies (5,19) in a general population-based sample. Lastly, our study also identifies some variants associated with lower serum calcium levels, one of which is a functional candidate gene variant that we confirm as associated with increased odds of kidney stone disease (6,20,21).

Rare mutations in the *CASR* gene can give rise to several Mendelian disorders: gain-of-function mutations can cause autosomal-dominant hypocalcemia (MIM #146200) or a form of Bartter syndrome, and loss-of-function mutations can cause neonatal severe hyperparathyroidism in the homozygous or compound heterozygous state (MIM #239200) and FHH, type I in the heterozygous state (FHH, MIM # #145980). In FHH, inactivation of CASR leads to inappropriate renal re-absorption of calcium and magnesium, resulting in hypercalcemia and hypermagnesemia (22). The fact that the minor G allele of rs17251221 was associated with both higher serum calcium and magnesium levels suggests that the causal variant underlying the association signal in our study results in reduced CASR function. Moreover, the association of the G allele with lower serum phosphate concentrations is consistent with

Table 2. Association of the lead SNP, rs17251221 (MAF 14%) with serum calcium concentration per copy of the minor G allele

	FHS	ARIC	CHS	Rotterdam	HABC	AGES	Meta-analysis
Imputation score	0.960	0.998	0.515	0.998	1.0	0.998	
P-value	1.5×10^{-4}	5.4×10^{-14}	0.01	0.004	4.8×10^{-4}	0.24	2.4×10^{-22}
β (SE)	0.051 (0.014)	0.067 (0.009)	0.057 (0.022)	0.065 (0.022)	0.070 (0.020)	0.031 (0.027)	0.061 (0.006)

HABC, Health ABC Study; SE, standard error; MAF, minor allele frequency. Imputation quality scores were calculated for each SNP as the ratio of observed dosage-variance to the expected binomial variance. Meta-analysis estimates for rs1801725 (A986S) were 0.058 (s.e. 0.006) per copy of the minor T allele ($P = 3.0 * 10^{-22}$).

a loss of function variant, as another role of the CASR is to reduce the inhibitory effect of PTH on renal phosphate re-absorption (23). The association of the G allele with higher PTH levels did not reach statistical significance. In comparison to the clinical chemistry observed among individuals with FHH, the directions of the associations were the same, but of smaller magnitude. For example, mean serum calcium levels among FHH patients have been reported as 11 mg/dl (2.75 mmol/l) (24), whereas mean serum calcium levels were higher with each copy of the G allele, but still within the reference range for serum calcium levels in our study.

An alternative explanation for our findings could be reduced expression of the CASR. In humans, the CASR is expressed in multiple tissues (http://www.genecards.org/). Genetic variants directly controlling CASR expression have not been reported so far (16). The lack of association we observed between rs17251221 or correlated variants ($r^2 > 0.5$) and CASR transcript levels supports this notion, although we did not have access to tissue-specific gene expression data sets from parathyroid or kidney.

The coding SNP rs1801725, which is highly correlated with rs17251221, leads to an alanine-to-serine substitution at position 986 in CASR (A986S). This amino acid change is predicted as tolerated in SIFT and PolyPhen and of uncertain functional significance (http://www.casrdb.mcgill.ca) (14,25). The A986S polymorphism has been detected among families with FHH (26) and in the general population (6,20,21). In agreement with the findings from the present study, these studies and others (27) have observed an association between the minor allele and higher levels of serum calcium. We extend the literature by demonstrating the robustness of these results in much larger samples, as well as demonstrating associations with serum magnesium and phosphorus levels.

In agreement with some previous studies of the correlated A986S variant, the G allele of rs1801725 was associated with lower BMD at the lumbar spine. Individuals with FHH usually have measures of BMD in the normal range (28), but lower BMD is observed in individuals affected by neonatal severe hyperparathyroidism, a more severe form of hypocalciuric hypercalcemia caused by *CASR* mutations. Here, increased levels of PTH lead to higher bone resorption. The lack of association with femoral BMD could be explained by the importance of genes specific to BMD at one of the two sites as observed in GWAS of BMD (13). On the other hand, the observed association with lumbar spine BMD should be interpreted with caution, as the *P*-value of 0.038 was of borderline statistical significance.

Our findings support the notion that there is a wide spectrum of genetic variation in CASR, with phenotypes that range from very mild to those not compatible with survival. Although the variants we observe are associated with mild alterations of clinical measures, it is possible that the effects may be more pronounced in individuals with disturbances of the calcium–phosphate metabolism. This will therefore be an interesting area of future research, as will be the pursuit of genomic regions with evidence for suggestive but not genome-wide significant association.

Our study has several limitations. The study included data only from individuals of European ancestry and is therefore of limited generalizability. We studied the association with total serum calcium levels rather than with corrected serum calcium levels. Inter-individual differences in the protein-bound fraction of calcium may therefore lead to some misclassification of the concentration of ionized serum calcium. However, sensitivity analyses suggested that this misclassification is non-differential with respect to genotype and the association with rs17251221 remained unchanged. The availability of PTH on only a subsample limited statistical power to evaluate an association. The primary locus identified by this GWAS is in high LD with the previous A986S variant. The causal variant underlying these observed associations remains to be determined; a search of publicly available databases [Ensembl, JASPAR (29) and ConSite (30)] investigating the region of the 17251221 or rs1801725 variants did not find evidence for the presence of any regulatory elements.

In addition to rare variants, common variants in the *CASR* gene exert an effect on serum calcium levels in the general population. The G allele at rs17251221 associates with similar but milder clinical features than the ones observed in individuals with autosomal-dominant FHH, namely hypercalcemia, hypermagnesemia and hypophosphatemia.

MATERIALS AND METHODS

Serum calcium indices

Serum calcium was measured as described in Study-specific methods.

Genome-wide genotyping platforms and imputation

Genotyping was conducted as described previously (15), summarized in Study-specific methods and detailed in Supplementary Material, Table S1. Quality control procedures were implemented on SNPs genotyped as part of the commer-

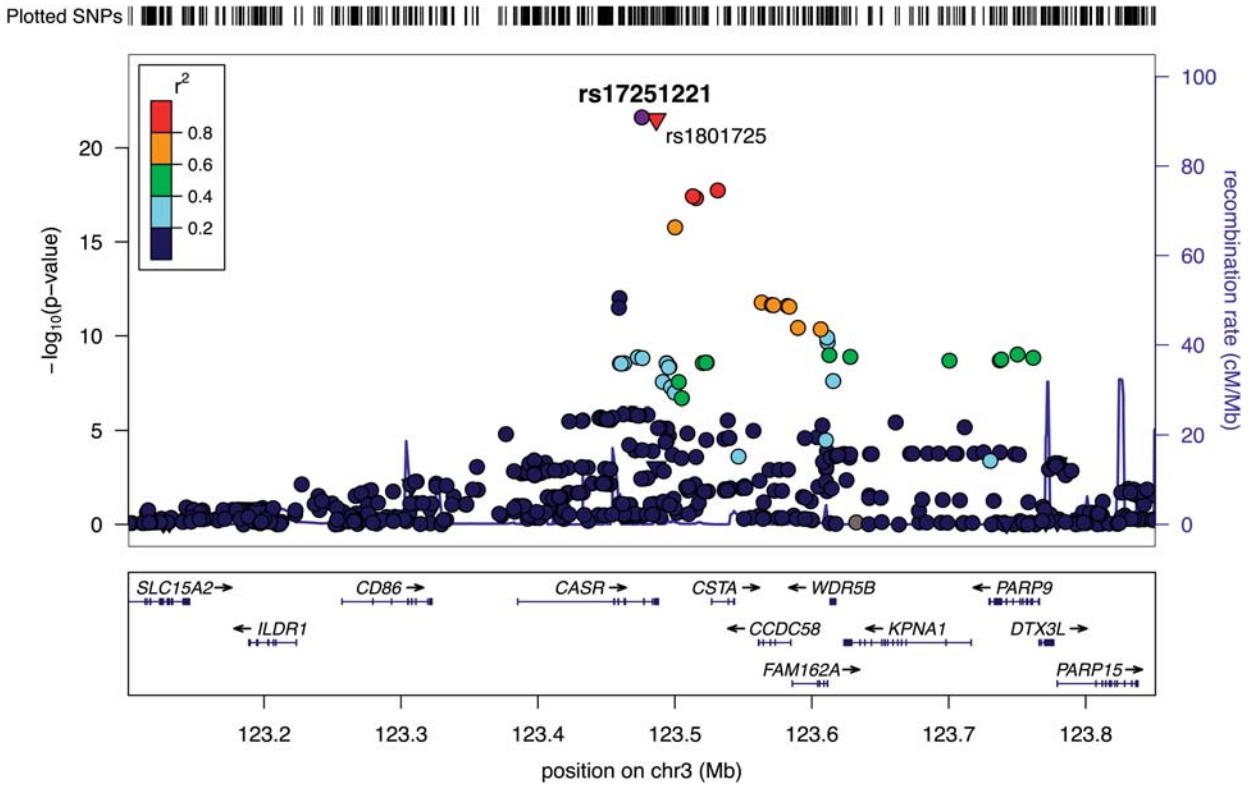

Figure 1. Regional association plot of the *CASR* locus. *P*-values are corrected for genomic control. Regional association plot has been graphed using the software LocusZoom (48).

cial genome-wide arrays and were then imputed to ~2.5 M HapMap CEU SNPs (Supplementary Material, Table S1).

Statistical methods for the meta-analysis

Details regarding the trait creation are described above. Each individual study performed a genome-wide association analysis of total serum calcium concentrations using linear regression; an additive genetic model was used. We adjusted for age, sex,-study center and principal components where applicable. The Framingham Heart Study accounted for relatedness.

We performed a fixed-effect meta-analysis of serum calcium–SNP association results from each study. Inverse-variance-weighted meta-analysis was performed using METAL (http://www.sph.umich.edu/csg/abecasis/Metal/index.html). To indicate genome-wide statistical significance, we used the threshold value of $<5 \times 10^{-8}$.

R software (version 2.9.0), the probABEL package (31) and PLINK (32) were used for data management, statistical analyses and graphing (33).

HERITABILITY AND PROPORTION OF VARIANCE EXPLAINED

Heritability was calculated in the Framingham Heart Study using a variance components method implemented in SOLAR v.1.4 as previously described (34). The proportion of serum calcium variance explained by rs17251221 was calculated based on the effect results from the meta-analysis and the standard deviation of serum calcium levels in the Atherosclerosis Risk in Communities (ARIC) Study as $r^2 = 2 * \mathrm{MAF} * (1-\mathrm{MAF}) * (\beta/\mathrm{SD})^2$.

Cross-trait associations

We examined our lead SNP in association with serum phosphate among 16 264 participants of the CHARGE cohorts that contributed to a meta-analysis of GWAS of serum phosphate levels (35). This meta-analysis data set for serum phosphate associations was generated excluding individuals with an estimated eGFR of <45 ml/min/1.73 m^2 (35). The association with PTH levels was examined among 1731 participants of the Cardiovascular Health Study (CHS). To be consistent with the analytical strategy for serum phosphate concentrations, the association with PTH was also evaluated excluding individuals with eGFR of <45 ml/min/1.73 m^2 and adjusted for age, sex and study center. The association with serum magnesium was evaluated in a data set from a meta-analysis of GWAS of serum magnesium levels among 15 366 participants of the CHARGE cohorts (36). As for serum calcium, the serum magnesium–SNP associations had been adjusted for age, sex and study center where applicable. Serum magnesium levels were measured in the individual cohorts using colorimetric methods. A potential alternative explanation for the association with serum magnesium levels could therefore be an assay artifact if the measurement of serum calcium and magnesium levels were to interfere. An

association with kidney stone disease was examined in a meta-analysis data set of the CHARGE cohorts [AGES, ARIC, FHS, HABC, Rotterdam Study (RS)]. Kidney stone disease was defined based on self-report and/or the abstraction of ICD-9 codes including a kidney stone disease diagnosis in any position from hospitalization records. Data for associations with BMD were available as an *in silico* lookup from a meta-analysis of femoral neck and lumbar spine BMD measured by DXA in 19,195 individuals of Northern European descent as part of the GEFOS Consortium (13). Participants of the RS as well as of a subset of the Framingham Heart Study contribute information to the GEFOS Consortium, so that approximately two-thirds of the participants in the GEFOS data set used for the lookup of the BMD association were individuals not contributing information to the CHARGE Consortium. The lookup of the other trait–SNP associations were conducted among studies participating in the CHARGE Consortium (7).

Study-specific methods

Age Gene/Environment Susceptibility-Reykjavik Study. The Age Gene/Environment Susceptibility (AGES)-Reykjavik Study is drawn from the Reykjavik Study (37); 5764 subjects were recruited between 2002 and 2006. Serum calcium was measured in serum on a Hitachi 912 device, using reagents from Roche Diagnostics and following the manufacturer's instructions. The Illumina 370CNV BeadChip array was genotyped on 3664 participants. No significant association with the first principal component (PC1) was observed.

Atherosclerosis Risk in Communities Study. The ARIC Study is a prospective population-based study that started in 1987–1989 when 15 792 adults aged 45–64 years from four US communities were enrolled (38). Participants for the current study included those with measures of serum calcium from visit 1. Total serum calcium was measured at ARIC visit 1 (1987–1989) using a calorimetric method on a DACOS analyzer (http://www.cscc.unc.edu/aric/visit/Clinical_Chemistry_Determinations.1_10.pdf). PC1 estimated from Eigenstrat (39) was associated with serum calcium levels and therefore included as an additional covariate. Sensitivity analyses were conducted in the ARIC Study, the largest contributing cohort, and included the correction of serum calcium levels corrected for the amount of total protein, the additional adjustment of the SNP–calcium association for serum phosphate concentrations and the association between SNPs and serum calcium levels before and after the exclusion of 258 individuals with CKD (defined as GFR estimated by the four-variable MDRD Study equation of <60 ml/min/1.73 m^2), as well as the association between rs17251221 and serum calcium and phosphate. Two sets of sensitivity analyses were conducted to compare the use of total calcium with that of corrected calcium. First, corrected calcium values were obtained using a regression-based formula correcting for individual serum albumin concentrations as described previously (40): corrected calcium (mmol/l) = total calcium (mmol/l) − [1.32 * serum albumin (g/dl)] + 1.928, where 1.32 is the coefficient for serum albumin and 1.928 the intercept obtained from a regression of serum calcium on albumin values. Second, corrected calcium levels were obtained using the correction formula: corrected calcium (mmol/l) = total calcium (mmol/l) + ([40 − serum albumin (g/dl)] * 0.025) (41).

Cardiovascular Health Study. The CHS is a longitudinal study of cardiovascular disease and stroke among adults aged 65 or older; participants were recruited at four field centers (Forsyth County, NC; Sacramento County, CA; Washington County, MD; Pittsburgh, PA) (42). African-American participants were excluded from this analysis since the other cohorts were predominantly Caucasian. Serum calcium was measured using indirect potentiometry on a Beckman-Coulter DXC Synchron clinical analyzer. The inter-assay coefficient of variation was 1.4%. Serum intact PTH was measured using a two-site immunoassay on a Beckman-Coulter DXI clinical immunoassay analyzer. The inter-assay coefficient of variation was 4.5%. No association with any principal components was observed.

Framingham Heart Study. In 1971, the Offspring Cohort of the Framingham Heart Study was enrolled (5124 participants); the methodology and design has been described (43,44). The present study consists of participants from the offspring cohort who attended the second exam (1979–1983). Serum calcium was measured using a colorimetric assay. We observed association with serum calcium and PC1 using Eigenstrat (39), and therefore included it in the analysis for association between genotype and serum calcium as a covariate.

Health ABC. The Health ABC Study began in 1997 as a community-based prospective cohort study with participants aged 70–79 recruited from Medicare listings in Pittsburgh, Pennsylvania and Memphis, Tennessee. The present analyses are limited to those Health ABC participants with self-reported race/ethnicity as European-American. Total calcium was measured in serum samples collected at the HABC year 2 visit with direct quantitative colorimetric determination using Stanbio Total Calcium LiquiColor Procedure No. 0500 (Stanbio Laboratory, Boerne, TX, USA). The inter-assay coefficient of variation was 2.2%.

Genotyping was performed by the Center for Inherited Disease Research (CIDR). No association with PC1 was observed.

Rotterdam Study. The RS is a population-based study from Ommoord, in the Netherlands, beginning in 1990–1993 (45–47). Serum calcium was measured at baseline visit in the RS with a colorimetric detection assay using the Hitachi 917 (Roche, Mannheim, Germany). No association with PCs was observed.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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