

## Relationship between Vascular Calcification and Bone Mineral Density in the Old-Order Amish

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**Abstract.** Vascular calcification and osteoporosis are common age-related processes that are influenced by both genetic and nongenetic factors. Whether common genes underlie these processes is not known. We measured coronary artery calcification (CAC), aortic calcification (AC), and bone mineral density (BMD) in 682 men and women from large Old-Order Amish families. We assessed the heritabilities of these traits and then evaluated, using variance decomposition procedures, whether variation in the traits was influenced by a common set of genes (i.e., pleiotropy). Significant heritabilities were detected for BMD of the femoral neck and spine (0.65, 0.63) and CAC and AC (0.43, 0.42). Mean BMD did not differ significantly across quartiles of either CAC or AC in either sex. In neither the total group nor any single subgroup (men, women, postmenopausal women) did any of the genetic or environmental correlations between BMD and vascular calcification achieve statistical significance. However, subjects with a history of cardiovascular disease (CVD) events had significantly lower BMD at the femoral neck compared to subjects who reported no prior history of CVD (age-, sex-, body mass index-, and family structure-adjusted  $P = 0.003$ ). We detected no evidence for shared genes affecting the joint distribution of bone and vascular calcification. However, our results do reveal a lower BMD in subjects with a prior history of CVD in the Old-Order Amish.

**Key words:** Bone mineral density — Vascular calcification — Pleiotropy — Amish — Cardiovascular disease

The tendency of cardiovascular disease (CVD) and osteoporosis to aggregate more often than would be expected by chance was first highlighted by Browner et al. [1], who observed that postmenopausal women with low bone mineral density (BMD) experienced a higher risk of

CVD mortality than women with higher BMD. Subsequent studies have reported inverse correlations between BMD and degree of coronary atherosclerosis [2, 3] and between rate of bone loss and progression of aortic calcification (AC) [4, 5], providing further support for a link between CVD and osteoporosis.

The mechanisms accounting for the observed associations between CVD and osteoporosis are unclear. The association may be only indirect, such that individuals having specific lifestyles or behaviors may be at risk for both disorders, or it may be more direct, with common biological processes contributing jointly to accelerated loss of bone and increased cardiovascular risk. Some, e.g., have proposed that atherosclerosis of the microvasculature supplying bone may promote bone loss, in contrast to the atherosclerosis occurring in large vessels that promotes mineralization of vascular tissue [6]. Further speculation is that these effects might be partially mediated by oxidized lipids and/or other inflammatory factors [7]. Other mechanisms may also be involved, including pathways involving osteoclast formation, as suggested by recent studies showing that osteoprotegerin (OPG)-deficient mice exhibit decreased BMD and elevated rates of arterial calcification [8].

Because calcification in the coronary and extracoronary arterial beds correlates with the extent of atherosclerotic lesions [9], coronary artery calcification (CAC) independently predicts angiographically defined coronary artery disease (CAD) [9] and CAC predicts future CAD events independently of other CVD risk factors in asymptomatic and symptomatic adults [10, 11]. Some epidemiological studies aimed at elucidating mechanisms between CVD and BMD have considered the potential link between vascular and skeletal calcification. In fact, some [4, 5, 12], but not all [13, 14], studies have reported that the degree of AC is correlated with

the extent of bone loss and/or risk of osteoporotic fractures. Most studies reporting significant associations between bone loss and degree of vascular calcification have been carried out in postmenopausal women [4, 12].

To help clarify the relationship between BMD and calcification in different vascular beds, we measured BMD and assessed extent of calcification in the coronary arteries and thoracic aorta in an adult population of men and women having a relatively homogeneous lifestyle. We then determined the relationship between BMD and vascular calcification. These individuals comprised large families, further allowing us to evaluate whether genes influencing variation in BMD also influenced variation in vascular calcification.

## Material and Methods

### Subjects

Subjects for this study were participants of the Amish Family Calcification Study (AFCS), a community-based study that was initiated in 2001 to identify the joint determinants of BMD and vascular calcification. Subjects were initially recruited into the AFCS on the basis of their prior participation in an earlier family study of BMD [15], although for practical considerations (e.g., to maintain rapport with the community) recruitment guidelines were modified to allow other interested individuals to participate. First- and second-degree relatives of these new participants were also invited to participate in the AFCS. Recruitment efforts were made without regard to CVD health status in this generally healthy population. Because vascular calcification occurs infrequently in young adulthood, only individuals aged 30 years and older were enrolled into the AFCS. The analyses presented in this report are based on AFCS participants examined from the start of recruitment in March 2002 through July 2005 ( $n = 682$ ). 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 that included assessment of potential risk factors for CVD and a medical history interview. This initial assessment was conducted 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 determination of lipid levels. Lipid concentrations were assayed by Quest Diagnostics (Baltimore, MD). Low-density lipoprotein cholesterol levels were calculated using the Friedewald equation [16]. Height and weight were measured using a stadiometer and a calibrated scale with shoes removed and in light clothing. Body mass index (BMI,  $\text{kg}/\text{m}^2$ ) was computed. Systolic (first phase) and diastolic (fifth phase) blood pressure measurements were obtained in triplicate using a standard sphygmomanometer with the subject sitting for at least 5 minutes and recorded to the nearest 1 mm Hg. Use of medications and current smoking status were assessed by self-report. Diabetes mellitus was defined as a fasting glucose  $\geq 126$  mg/dL or use of diabetes medications. The lipids, lipoproteins, mean blood pressure, medication use, and history of smoking were used to describe the sample.

### Assessment of Bone Mass and Vascular Calcification

BMD at the lumbar spine (anteroposterior position) and hip were measured by dual-energy X-ray absorptiometry. BMD

were determined by dividing the total bone mineral content (g) by the projected area of the region scanned ( $\text{cm}^2$ ). BMD images were assessed by a registered nurse certified in clinical densitometry by the International Society for Clinical Densitometry. Vertebrae containing compression fractures and structural anomalies (including postoperative changes and/or sclerosis) and those with a T-score difference of 1.0 or more from the L1–L4 mean [17] were excluded from analysis. Coefficients of variation, determined annually by three sequential measures on one day for each of 15 individuals, were 0.90% for total hip and 0.71% for the spine (L1–L4). For this report, we restricted our analyses to BMD measurements obtained at the spine and femoral neck.

Electron beam computed tomographic scans were performed on an Imatron (Baltimore, MD) C-150 scanner. CAC scanning was performed using a standard protocol that included 30–40 contiguous transverse slices (3 mm) between the aortic root and the apex of the heart, gated to 80% of the R-R interval (the cycle between two 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 of  $> 130$  Hounsfield units in more than three contiguous pixels ( $> 1 \text{ mm}^2$ ). CAC was quantified using the Agatston method, which incorporates both density and area [18]. The sum of the scores in the left main, left anterior descending, circumflex, and right coronary arteries was considered the CAC score. AC from the upper thoracic region was quantified also using the Agatston method, and the sum of all the AC lesions was considered the AC score. All scans were scored by a single experienced cardiologist and reviewed for extracardiac findings by a single experienced radiologist.

### Statistical Methods

Because the distribution of CAC and AC scores was positively skewed and not all subjects had detectable calcification, the scores were natural log-transformed after adding 1. Age- and sex-adjusted (in total sample) or age-adjusted (in each subgroup) CAC and AC scores were calculated from linear regression analysis, and standardized residuals were obtained from the regression model. The standardized residuals were approximately normally distributed. Those standardized residuals that were extreme ( $n = 3$ ) were “Winsorized” by replacing these values above the 99th percentile with the value of the 99th percentile.

Inferences about genetic effects were estimated from the phenotypic similarities between individuals and their type of relationship based on basic quantitative genetic principles. Univariate and bivariate variance decomposition analyses were performed using the Sequential Oligogenic Linkage Analysis Routines (SOLAR) software program [19]. Initially, we modeled variation in vascular calcification and BMD as a function of measured environmental covariates, additive genetic effects (or heritability), and a residual error component. Covariates for BMD heritability analysis were age, age<sup>2</sup>, sex, and BMI. BMI was used as a covariate on age- and sex-adjusted CAC and AC scores. Maximum likelihood methods were used to estimate the covariate and genetic effects simultaneously. The significance of particular components can be assessed by comparing the likelihood of a model containing the component of interest to that of a model in which the effect of the component is constrained to zero. The full and restricted models are then compared by likelihood ratio test, which produces a test statistic that is asymptotically distributed as a  $\chi^2$  distribution.

We initially examined the relationship between BMD and vascular calcification by comparing BMD values across quartiles of vascular calcification in each sex. Vascular calcification quartiles were ranked using the standardized residual of log-transformed calcification score. The trend in BMD across vascular calcification categories was evaluated by modeling BMD

**Table 1.** Clinical characteristics (mean, SD) of the study population

	Men	Women	Age-adjusted <i>P</i>
<i>n</i>	301	381	
Age (years)	54.1 (13.0)	54.8 (13.2)	0.46 <sup>b</sup>
BMI (kg/m <sup>2</sup> )	27.3 (4.5)	29.1 (5.7)	< 0.0001
Systolic blood pressure (mm Hg)	119.5 (14.0)	119.7 (18.6)	0.85
Diastolic blood pressure (mm Hg)	73.5 (8.9)	70.6 (8.8)	< 0.0001
Cholesterol (mg/dL)	205.9 (36.6)	218.1 (43.7)	0.0002
Triglyceride (mg/dL)	85.6 (55.3)	95.6 (67.2)	0.046
HDL-C (mg/dL)	53.0 (14.1)	60.5 (15.1)	< 0.0001
LDL-C (mg/dL)	135.7 (33.1)	138.9 (40.2)	0.39
Current smoker	18.3%	0.3%	< 0.0001
Cholesterol medications	5.8%	4.6%	0.49
Blood pressure medications	6.5%	7.9%	0.60
Diabetes medications	1.5%	2.0%	0.60
Spine BMD (g/cm <sup>2</sup> )	0.95 (0.13)	0.91 (0.15)	0.004
Femoral neck BMD (g/cm <sup>2</sup> )	0.84 (0.12)	0.81 (0.14)	0.001
Self-reported CVD events <sup>a</sup>	15.0%	6.3%	0.0002
Presence of CAC	58.5%	42.5%	< 0.0001
Presence of AC	56.8%	57.0%	0.98
Median CAC (25%, 75%)	13 (0, 206)	0 (0, 34)	< 0.0001
Median AC (25%, 75%)	49 (0, 719)	37 (0, 1326)	0.82

<sup>a</sup> Includes myocardial infarction, stroke, coronary surgery, blocked arteries, positive angiogram, and carotid surgery (*n* = 68)

<sup>b</sup> *P* value for age is unadjusted

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol

as the dependent variable and vascular calcification quartiles, age, and BMI as independent (predictor) variables.

More detailed analyses were then conducted to assess the genetic and environmental correlations between BMD and vascular calcification since correlations attributable to genes alone or to environmental factors alone could be obscured when considering the overall (phenotypic) correlations between traits only. We extended the univariate genetic analyses described above to evaluate the bivariate relationship between BMD and vascular calcification. The joint bivariate phenotype of BMD and vascular calcification was modeled as a linear function of the individual's phenotypic values, the population means, the additive genetic values, and environmental effects [20]. The covariates included in the model were age, age<sup>2</sup>, sex, and BMI. The genetic and environmental variance-covariance matrices were calculated and genetic and environmental correlations estimated. A model with all parameters was compared with a model in which the genetic correlation was constrained to zero. A genetic correlation whose value differed significantly from zero implied that a common set of genes contributed to the variations in the measures of the two traits.

We further compared mean BMD between the 69 subjects with self-reported CVD events and a control group of subjects reporting no history of CVD events. Because the subjects with a history of CVD events tended to be older (mean age 64.5 years) than those without, we selected for control subjects all those who reported no history of CVD events and were 50 years of age or older (*n* = 353, mean age 62.1 years). The effect of CVD history on BMD was then assessed under the variance component model with adjustment for sex, age, BMI, and genetic relationships among these 422 subjects.

## Results

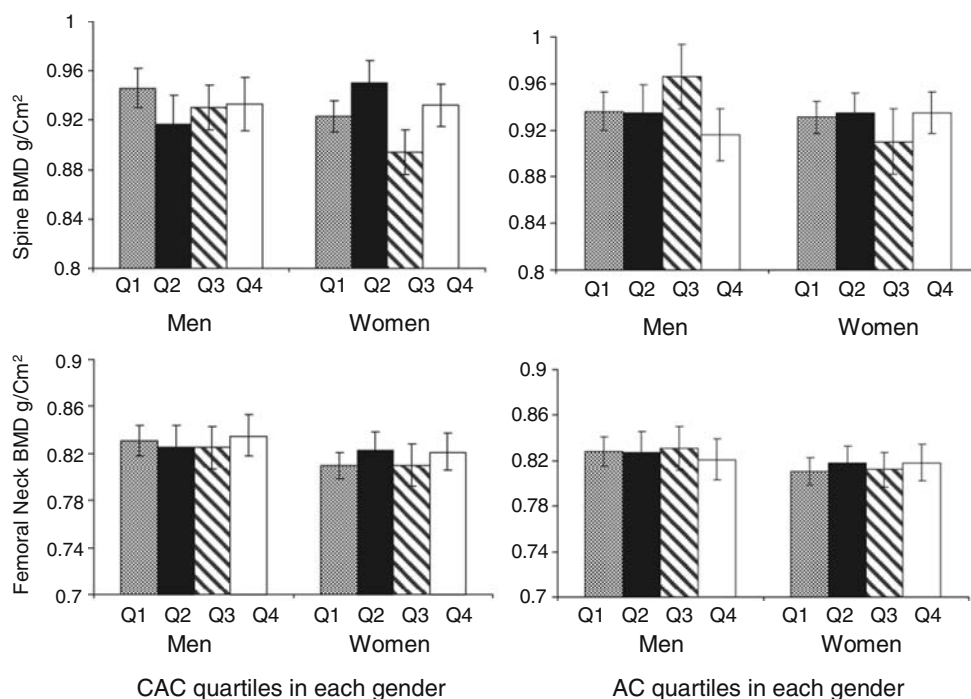
The 682 study subjects included individuals from 381 sibships, ranging in size from 1 to 11. Additional relationship types were identified by linking nuclear families together to form larger pedigrees through their unexamined parents. These more extensive pedigrees were utilized to provide additional power for the genetic

analyses. A total of 93 pedigrees could be formed, ranging in size from 2 to 48 examined individuals and representing 652 sib pairs, 272 parent-offspring pairs, 480 avuncular pairs, and 229 first cousin pairs.

Clinical characteristics of study subjects, stratified by sex, are presented in Table 1. The study sample included 301 men (mean age 54.1 years, range 30–85) and 381 women (mean age 54.8 years, range 30–90), 186 of whom were postmenopausal. One postmenopausal woman reported use of an osteoporosis medication (bisphosphonate). Two individuals (one man and one woman) self-reported a nontraumatic hip fracture in the past. The proportion of subjects reporting current usage of blood pressure-lowering and cholesterol-lowering medications was low. In general, men had higher BMD at each site than women (*P* = 0.004 at spine and *P* = 0.001 at femoral neck). The prevalence of CAC was markedly higher in men compared to women (*P* < 0.0001), although there was no sex difference in the prevalence of AC (*P* = 0.98).

We initially assessed the association between vascular calcification and BMD by comparing mean BMD across quartiles based on the distribution of the transformed and adjusted calcification scores. As shown in Figure 1, mean BMD did not differ significantly across quartiles of CAC or AC in either sex.

We next assessed the relationship between BMD and vascular calcification in more detail by considering the more specific hypothesis of whether there might be correlations between these traits attributable specifically to either shared genes (genetic correlations) or shared non-genetic factors (environmental correlations). Conceptually



**Fig. 1.** Spine and femoral neck mean BMD levels across CAC and AC quartiles in each gender. BMD levels were adjusted for age, BMI, and family structure. Bars represent means and standard errors.

**Table 2.** Heritability ( $h^2 \pm SE$ ) of vascular calcification and BMD

Traits	$h^2 \pm SE$	$P$
CAC <sup>a</sup> ( $n = 682$ )	$0.43 \pm 0.09$	$1.04E-08$
AC <sup>a</sup> ( $n = 652$ )	$0.42 \pm 0.09$	$1.00E-07$
Spine BMD <sup>b</sup> ( $n = 652$ )	$0.63 \pm 0.11$	$9.30E-14$
Femoral neck BMD <sup>b</sup> ( $n = 651$ )	$0.65 \pm 0.08$	$2.18E-09$

<sup>a</sup> Age- and sex-adjusted score, adjusted for BMI

<sup>b</sup> Covariates include age, age<sup>2</sup>, sex, and BMI

ally, this analysis corresponds to assessing the heritability of each trait in the pair and then considering whether the genetic contributions to each trait are correlated (or conversely whether the nongenetic contributions to each trait are correlated). The heritability estimates for BMD and the vascular calcification traits are presented in Table 2. The heritabilities of CAC and AC scores were 0.43 and 0.42, while those of the spine and femoral neck BMD were 0.63 and 0.65. Estimates varied modestly for subgroups defined by sex or menopausal status (data not shown). Age, age<sup>2</sup>, sex, and BMI were the covariates for BMD heritability analysis and accounted for 20% of the variance for spine BMD and 36% for femoral neck BMD.

The genetic and environmental correlations between BMD and the vascular calcification traits are presented in Table 3. These correlations were computed for the entire sample (adjusting for age, age<sup>2</sup>, sex, and BMI) and separately for men and women and for postmenopausal women to allow for the possibility that the correlation is expressed predominantly in one group. In neither the total group nor any single subgroup (data not shown) did any of the genetic or environmental correlations between

BMD and vascular calcification achieve statistical significance. Parallel analyses were conducted on the subset of individuals in whom detectable vascular calcification was present (318 subjects had detectable CAC and 348 subjects had detectable AC), and in none of these analyses were significant genetic or environment correlations observed (data not shown).

We then hypothesized that an association might exist between BMD and CVD status. To test this hypothesis, we compared mean BMD between the 69 subjects with self-reported CVD events and a control group of similarly aged subjects reporting no history of CVD events. Reported CVD events included myocardial infarction, stroke, coronary surgery, blocked arteries, positive angiogram, and carotid surgery. The control group consisted of the 353 subjects aged 50 years and older who reported no history of CVD events. These analyses, adjusted for sex, age, BMI, and genetic relationships, revealed that subjects with a CVD history had significantly lower femoral neck BMD ( $P = 0.003$ ) (Table 4). The magnitude of effect was similar in men and women, with no statistical evidence for a sex-CVD history interaction on BMD. We repeated these analyses after removing subjects with a CVD history and taking medications that might have a potentially adverse effect on bone health (e.g., antiandrogen) ( $n = 1$ ). Results were essentially unchanged.

## Discussion

Despite a number of epidemiological reports suggesting an association between CVD and osteoporosis and/or

**Table 3.** Genetic and environmental correlations between vascular calcification and BMD levels

	$\rho_{\text{gene}}$	$P$	$\rho_{\text{env}}$	$P$
CAC <sup>a</sup> and BMD <sup>b</sup>				
Spine	0.088 ± 0.143	0.54	-0.105 ± 0.132	0.42
Femoral neck	0.009 ± 0.156	0.95	0.022 ± 0.142	0.88
AC <sup>a</sup> and BMD <sup>b</sup>				
Spine	-0.108 ± 0.150	0.47	0.071 ± 0.132	0.59
Femoral neck	-0.048 ± 0.163	0.7	0.037 ± 0.147	0.80

<sup>a</sup> Age- and sex-adjusted score

<sup>b</sup> Covariates include age, age<sup>2</sup>, sex, and BMI

**Table 4.** Comparison between subjects with and without CVD history (mean, SD)

	With CVD history ( <i>n</i> = 69)	Without CVD history ( <i>n</i> = 353)	$P$
Age (years)	64.5 (12.4)	62.1 (8.7)	0.057
Percentage of male ( <i>n</i> )	65.2% (45)	40.2% (142)	0.0001
BMI (kg/m <sup>2</sup> )	28.4 (5.9)	28.9 (5.2)	0.48
Spine BMD (g/cm <sup>2</sup> )	0.89 (0.18)	0.90 (0.15)	0.40 <sup>a</sup>
Femoral neck BMD (g/cm <sup>2</sup> )	0.75 (0.15)	0.79 (0.13)	0.003 <sup>a</sup>
Presence of CAC	76.80%	67.10%	0.11
Presence of AC	85.30%	81.90%	0.50
Median CAC (25%, 75%)	255 (6.8, 1,281)	24 (0, 219)	< 0.0001
Median AC (25%, 75%)	2,586 (358, 6,817)	428 (31, 1,866)	< 0.0001

<sup>a</sup> Adjusted for age, sex, BMI, and family structure

BMD [2, 4, 5], the mechanisms accounting for the observed associations remain unclear. The possibility of an underlying mechanism linking calcification of bone and vasculature is an attractive one, especially since some cell types represented in bone and vasculature, such as chondrocytes and smooth muscle cells, share the same precursor (mesenchymal progenitor cell). Moreover, vascular cells have the potential for osteoblastic differentiation. Parhami and colleagues [7] have, in fact, suggested that a common underlying stimulus (oxidized lipids) might have opposite effects on vascular cell and bone cell differentiation by inducing vascular cell calcification and inhibiting bone cell differentiation. Regulation of OPG, a molecule inhibiting osteoclast formation, or regulation of its signaling pathways has been implicated in the pathogenesis of vascular calcification based on recent studies showing that OPG-deficient mice exhibit both decreased BMD and elevated rates of medial calcification [8]. However, the relevance of this model to atherosclerotic disease in humans is unclear since medial calcification is typically thought to be induced through metabolic, rather than atherosclerotic, pathways and no atherosclerotic plaques were, in fact, seen in these OPG-deficient mice.

Despite much current interest in the potential link between CVD and osteoporosis, the epidemiological evidence supporting this relationship is equivocal. While an association between subclinical measures of atherosclerosis, including vascular calcification, and osteoporosis/bone loss has been observed in a number of populations [2, 4, 5, 12, 21–24], it has not been observed in many others [13, 14, 25–30], including this Old-Order Amish population. Moreover, in subgroup analyses, we failed to detect an association in men only, women only, or postmenopausal women.

A unique feature of our family-based design in this genetically closed homogeneous population was our ability to address the more specific hypothesis of whether shared genes and/or shared environmental factors might influence the joint variation in both BMD and vascular calcification. Joint determinants due entirely to genes could be obscured if phenotypic variation due to environmental factors was large or if correlations between BMD and vascular calcification due to environmental factors (e.g., BMI) acted in the opposite direction from those due to genetic factors. In our study, the heritability of both BMD and vascular calcification was substantial ( $h^2 = 0.63$ – $0.65$  for BMD and  $0.42$ – $0.43$  for vascular calcification), with both estimates on the same order of magnitude as that reported in other populations [31–33]. However, our bivariate genetic analyses revealed no evidence that genes influencing variation in BMD measures also influenced variation in vascular calcification measures. Again, this was true both for the overall population and for the sex-specific subgroup analyses.

It is challenging to reconcile the differences in the literature between the positive correlations between

BMD and measures of subclinical atherosclerosis reported in some prior studies [2, 4, 5, 12, 21–24] and the lack of correlation reported in this and other populations [13, 14, 25–30]. It is possible that differing results from some of these studies may be partly due to context-dependent phenotype expression. For example, susceptibility alleles jointly affecting BMD and vascular calcification may be masked in some studies by the presence of protective lifestyle factors. The Old-Order Amish, e.g., are reported to have higher hip BMD than non-Amish whites and a lower incidence of hip fracture. This may be partly due to their higher level of physical activity and reduced frequency of some risk factors such as alcohol consumption, smoking, and prescription drug use [15]. In the absence of these risk factors, calcification-inducing genes may have a lower penetrance.

Also, there may be an underlying association between CVD and osteoporosis, but it may be only indirectly related to vascular calcification. Bone and vascular health are both mediated by a variety of common factors, including sex hormones, calcium-regulated hormones, vitamin K, homocysteine, lipid oxidation products, and other unknown factors [6]. Some of the associations reported previously have been observed in postmenopausal women [4, 5, 12, 12, 21, 23, 24]. Bone loss and progression of calcification in this group may be a consequence of independent mechanisms common to the aging process in general or secondary to other common factors, e.g., estrogen deficiency. Estrogens play a role in the protection against both CVD and osteoporosis through their effects on cytokines, such as interleukin-6, tumor necrosis factor  $\alpha$ , and OPG. An increase in these inflammatory cytokines and a decrease in OPG induced by lack of estrogens may be one of the common mechanisms mediating both bone loss and atherogenesis [reviewed in 34]. Nonetheless, we failed to detect a correlation in our data between BMD and vascular calcification even when our analyses were restricted to postmenopausal women aged 50 years and older.

Perhaps the most intriguing observation from our study is the lower BMD we observed at the hip, although not spine, in subjects with a history of CVD events compared to subjects reporting no prior history of CVD. While one cannot rule out the possibility that this association was observed only by chance in our population, it is nonetheless consistent with some of the literature suggesting that individuals with CVD or at high risk of CVD are more likely to experience low bone density and abnormal bone turnover [35–38], including the recent study by Kenny et al. [38] reporting significantly lower femoral BMD in patients with heart failure compared to healthy controls. In fact, it may be relevant to note that most of the previously reported cross-sectional studies in which no associations were observed between BMD and vascular calcification were con-

ducted in unselected and relatively healthy populations [14, 26, 28–30]. We investigated this theme further by evaluating whether subjects at high risk of CVD by virtue of their Framingham risk scores had lower BMD compared to age- and sex-matched controls but found no evidence for a correlation (data not shown). Nevertheless, it is possible that early CVD initiates some changes (possibly inflammation-related) that in turn contribute to acceleration of bone loss. It is also possible that clinical CVD leads to a decrease in physical activity, which in turn leads to an acceleration of bone loss.

In summary, we did not observe an association between BMD and vascular calcification in our overall sample of relatively healthy Amish individuals, although a statistically significant association was observed between BMD and history of CVD. Our analyses revealed BMD and vascular calcification to be moderately heritable but did not suggest a common set of genes contributing to both vascular calcification and BMD. Further research is necessary to confirm the underlying genetic basis for both traits.

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