GENETIC DETERMINANTS OF BONE MINERAL DENSITY IN MEN: A CANDIDATE GENE APPROACH TO STUDYING A COMPLEX TRAIT

by

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Osteoporosis is commonly considered a women's health problem, but is also a significant health concern for older men. Less is known about the predictors of osteoporosis and bone mineral density (BMD) in men. The aging of the population and expected increase in osteoporosis prevalence makes understanding the determinants of BMD of great public health importance.

Genetics is an important determinant of BMD, but little is known about specific loci associated with BMD in men and even less is known about the genetic influences on volumetric BMD (vBMD). With this in mind, we investigated 4108 single nucleotide polymorphisms (SNPs) in 383 candidate genes for their association within a population of older Caucasian men. In addition, we investigated 148 of these SNPs in 28 WNT pathway genes for gene-gene interactions and gene-environment interactions with physical activity and body weight.

We identified several SNPs that were associated with lumbar spine and femoral neck integral vBMD and explained 3.5% and 1.7% of the phenotypic variation in these traits, respectively. SNPs in two genes, adenomatous polyposis coli (APC) and the homeo box A gene cluster (HOXA) were associated with integral vBMD at both the femoral neck and lumbar spine. Analysis of cortical and trabecular vBMD at the femoral neck identified SNPs that explained 1.8% and 4.0% of the phenotypic variance, respectively. None of the SNPs for cortical vBMD were associated with trabecular vBMD. Statistically significant gene-gene and gene-environment interactions were also identified. Of note, statistically significant interaction effects of SNPs in

the low density lipoprotein receptor-related protein 5 and physical activity level on integral vBMD at the femoral neck and lumbar spine were identified.

Although additional work is needed to confirm and extend these findings we identified a number of novel associations for vBMD in older Caucasian men. Our results suggest the presence of genetic loci that are skeletal-site specific and specific to either cortical or trabecular bone. Additionally, these findings underscore the importance of evaluating genetic variation in the context of other genes and environmental factors.

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1.0 DISSERTATION OVERVIEW AND OBJECTIVES

Osteoporosis, a low bone mass condition that usually manifests with fragility fracture, is a global public health burden and serious concern for older adults. Though more common in women, this condition is also present in men and can lead to permanent disability and death.

In order to understand the factors that make an individual more susceptible to osteoporotic fracture, researchers often explore the correlates of BMD because of its correlation with bone strength and clinical utility[1-5]. Though many demographic characteristics and environmental factors influence BMD, genetic factors also appear to be very important. Family history is a significant and established predictor of osteoporosis[6] and heritability studies indicate that as much as 85% of the phenotypic variance in BMD may be explained by genetic variants[6-10].

While genetics has been established as an important factor in determining BMD, the relationship is complex and many questions remain to be answered. To address some of these unresolved questions we sought to investigate: what genes are skeletal site specific, what genes are specific to the cortical and trabecular bone compartments, and if gene-environment or gene-geneinteractions among components of the WNT signaling pathway (an important biological pathway in skeletal development and regeneration) are significantly associated with BMD in a sample of older men from the Osteoporotic Fractures in Men Study (MrOS).

1.1 SPECIFIC AIMS

The aim of *Research Article 1* was to explore the role of genetic variation in determining integral volumetric bone mineral density (vBMD) of the femoral neck and lumbar spine in older Caucasian men. To achieve this aim we tested for shared and skeletal site specific genetic effects in a two-phase candidate gene study of older men from the MrOS population study. In addition, we determined the amount of variation explained by significant genetic associations for vBMD.

The aim of *Research Article 2* was to identify genetic associations for cortical and trabecular vBMD of the femoral neck in older Caucasian men. We evaluated if these genetic associations were unique to either the cortical or trabecular bone compartment, or were associated with both bone compartments. In addition, we determined the amount of variation explained by significant genetic associations for the cortical and trabecular vBMD traits.

Research Article 3 investigated genotype-genotype interactions among genes in the WNT signaling pathway and also investigated selected interactions between genotype and "environmental" factors. Specifically, we investigated interaction effects on vBMD between SNPs in WNT pathway genes and physical activity as well as between SNPs in WNT pathway genes and body weight.

2.0 INTRODUCTION

2.1 OSTEOPOROSIS AND BONE MINERAL DENSITY

2.1.1 Osteoporosis Epidemiology

Osteoporosis is a condition of the skeletal system marked by low bone mass, bone fragility and susceptibility to fracture. Since this condition is generally asymptomatic until a fracture occurs, revised definitions of disease have been developed that incorporate both fragility fractures and low values of bone mineral density (BMD) into the disease definition[1-3].

In the United States, approximately 10 million people over age 50 have osteoporosis and 33.6 million more have low bone mass (also referred to as osteopenia)[4, 11]. Worldwide, osteoporosis is also a problem with as many as 30% of Caucasian women having osteoporosis at the hip[5]. Although BMD is used in defining osteoporosis, fragility fractures are the clinically significant event in the disease. Although fracture risks vary greatly in different regions of the world, fragility fractures are incredibly common. In the United States 40% of white women and 13% of white men will experience a fragility fracture in their lifetime[2, 5, 12]. More specifically, the lifetime risk of hip, spine and wrist fractures at age 50 is 18%, 16% and 16% respectively for white women and 6 %, 5% and 3% for white men (respectively)[12]. Estimates from 2000 demonstrate that fractures are also a problem worldwide with an estimated 9 million

osteoporotic fractures occurring, of which 1.6 million were hip fractures and 1.4 million were vertebral fractures[13, 14].

Although osteoporosis is a heterogeneous condition, epidemiologic investigation of disease reveals prevailing trends. The first pattern seen is with age. BMD increases with age until approximately age 30 and then begins to decline[5]. Osteoporosis prevalence markedly increases with an approximately seven-fold higher prevalence of disease being observed at age 80 compared to age 50[11, 15]. Finally, age trends are seen in fracture incidence. While many fractures occur in childhood, the non-traumatic, fragility fractures associated with osteoporosis are more common at older ages. Specifically, beyond age 50, hip fractures generally increase steadily until age 75 and then decrease[14]. In contrast, a greater number of wrist and spine fractures occur around age 50 an then slowly decrease with advancing age[14].

Gender differences in osteoporosis also exist. After achieving peak bone mass, women have greater bone loss with age than men[16, 17]. Studies of BMD using dual energy x-ray absorptiometry (DXA) indicate that men have higher BMD than women, however there are many methodological considerations and these will be discussed further in section 2.1.5 [17-19]. Fracture rates are higher in women than in men, which is likely a consequence of the higher rates of bone loss, weaker structural properties of bone and the greater likelihood of falling observed in women[17].

There is also a "clustering" phenomenon of fractures, meaning that the occurrence of one fracture makes it more likely that a participant will have other fractures[5, 17, 20]. In the Study of Osteoporotic Fractures, having had a vertebral deformity at baseline was associated with a 5-fold increased risk of additional vertebral deformity, a 2.8-fold increased risk in hip fracture and a 1.9-fold increased risk in any non-vertebral fractures[21]. Additionally, a population study of

residents in Rochester, Minnesota found that wrist fractures after age 35 were predictive of future hip fracture and vertebral fracture[22].

2.1.2 Osteoporosis Burden and Public Health Significance

Though low BMD is not a life threatening condition by itself, increased mortality after osteoporotic fracture has been observed. Hip and vertebral fractures are associated with excess mortality within 5 years of fracture and there are even cautious findings of excess mortality following wrist fracture[20, 23]. Worldwide, the estimated disability-adjusted life-years (DALYs) lost was 5.8 million[14]. Hip fractures alone account for 740,000 of deaths and 1.75 DALYs lost[14].

Morbidity following fracture is well documented. Among other things, vertebral fractures are associated with height loss, kyphosis, back pain, fatigue, social isolation, decreased physical activity and negative psychological sequelae[24]. Hip fractures often result in pain, permanent disability, loss of mobility, negative social and psychological functioning and the requirement of long-term nursing care[17, 20, 23].

Globally, osteoporosis makes up 0.83% of the non-communicable disease burden[14]. In the United States, direct healthcare expenditures including hospital and nursing home costs were estimated to be $17 billion in the year 2000. In the United States, an individual is estimated to have an additional $21,000 in medical costs the first year after a hip fracture and an additional $1,200-$3,600 excess costs following clinical vertebral fracture[12, 20]. Although much attention has been paid to the economic costs of hip fracture, other fracture types also make up a substantial proportion of the United States' health care expenditures[25].

The public health burden of osteoporosis is not inconsequential, but the aging of the population worldwide will further increase this burden. Between 1990 and 2050, the number of individuals over 65 is expected to increase five-fold worldwide; all other things being equal, the number of hip fractures will reach 6.3 million. A study by Burge et al. estimates that annual fracture costs in the United States will increase to 25.3 billion dollars by 2025 which is a 49% increase from 2005[26]. Such substantial increases will surely burden the health care system not only in terms of monetary resources expended, but also in terms of hospital and nursing home beds utilized.

2.1.3 Osteoporosis in Men

Osteoporosis is traditionally thought of as a problem of older women, and indeed, women make up the largest proportion of the absolute number of osteoporotic fractures in the United States and the world[17]. However, about one-third of all hip fractures and one half of all spine fractures occur in men which is a substantial number and a considerable public health burden[27].

As previously mentioned, the aging of the population is expected to markedly increase osteoporosis burden worldwide, and some of the greatest increases will be seen in males and the oldest-old (80+ years of age)[28]. This will likely mark a greater increase in osteoporosis in men not only because of the larger male population, but also because fractures in men typically occur later in life than in women. For example, though men have a lower fracture incidence across the lifespan, the incidence of spine fractures begins to increase dramatically in men at age 75 and by around age 80, men and women have approximately the same incidence rates[27].

There is evidence that the burden of OP for an individual is higher if that individual is a man. Specifically men are 3 times more likely to die following a hip fracture than women, and utilize 4% more health care dollars than women following a hip fracture[27].

2.1.4 Bone Mineral Density as a Correlate for Osteoporosis

Using fractures as the definition for osteoporosis has limited clinical utility. The goal of defining osteoporosis is to identify at risk individuals before a fracture occurs, when prevention strategies and therapeutics can be utilized[4].

Bone strength or stiffness cannot be directly measured *in vivo*, so another highly correlated clinical measure, bone mineral density (BMD), is used[5]. BMD, or the amount of bone mineral within an anatomically defined area, has been historically measured by a radiographic technique called dual-energy x-ray absorptiometry (DXA). DXA uses x-ray beams of two different energy levels; one that is absorbed by soft tissue and one that is absorbed by calcified tissue[29]. These two energy beams allow the absorbance from the soft tissue component to then be removed from the total absorbance to get a value for bone mineral content. This measure of bone mineral content is subsequently divided by the cross-sectional area of the anatomical region to obtain a measure of bone mineral density expressed as grams/cm^2.

BMD is not only correlated with bone strength, but has also proven to be clinically useful in identifying those at risk of fracture[30]. BMD has proven to be highly associated with fracture risk, even after adjusting for other lifestyle, anthropometric and medical factors[31]. In white women, a one standard deviation decrease in BMD is associated with anywhere from a 1.2 to 2.6 times higher risk of fracture[32-36]. Although less is known about the relationship in

men, studies indicate that a one standard deviation decrease in BMD is associated with between a 1.4 and 3.7 times higher risk of fracture[32-34, 36].

Analysis of specificity and sensitivity of DXA measures, an important factor in determining clinical utility, has been assessed by receiver operating curves (ROC) that plot the sensitivity vs. 1-specificity and determine how much better a test does than chance (using a measure of the area under the ROC curve or AUC). Specifically, AUCs of 0.69-0.72 were observed for areal BMD (g/cm^2) at the hip indicating that femoral neck BMD is a useful, but not perfect clinical measure[34, 35]. Furthermore, analysis using ROC curves indicates that femoral neck and trochanteric BMD are substantially better predictors of fracture than BMD for the whole body or Ward's triangle[35].

2.1.5 Volumetric Bone Mineral Density

A true measurement of bone density would express bone mineral content over bone volume, but DXA is a 2-dimensional assessment and can only measure cross-sectional area and not the volume of bone. Unlike DXA, which computes areal BMD, other measurement techniques can assess volume and provide volumetric bone mineral density (vBMD) measures. Quantitative computer tomography of the central skeleton (QCT) or the periphery (pQCT) is being used more frequently in epidemiological studies to measure vBMD. Furthermore, in vivo evidence indicates that QCT may be a more appropriate correlate of bone strength than DXA. Specifically, when excised femurs were mechanically tested for their breaking strength, measurements from QCT were better predictors of failure load than DXA[37].

Areal BMD by DXA is a two-dimensional technique that can also be confounded by bone size differences between individuals. Specifically, DXA tends to overestimate BMD in larger

bones[38]. This is particularly problematic when evaluating osteoporosis between individuals of different body size (e.g., comparisons of men and women). DXA BMD measures show markedly higher BMD in men, but when vBMD, which is not confounded by bone size, is assessed these gender differences are less apparent[39].

QCT and pQCT measures can also provide more detailed bone measurements. For example, bone is made up of two types of tissue; compact or cortical bone that makes up the outer layer and trabecular bone (also called cancellous bone) that makes up the spongy component on the interior of bones. With QCT, the cortical and trabecular bone compartments can be resolved separately unlike with DXA.

The clinical utility of vBMD is not yet fully understood. However, initial findings from the Osteoporotic Fractures in Men Study (MrOS) demonstrate that having a low trabecular vBMD, a low percentage of cortical vBMD or smaller cross-sectional area at the femoral neck is predictive of hip fracture in older men[40]. Although these findings indicate that QCT may be important in the prediction of fracture, they were not superior to DXA BMD measurement and when used in conjunction with DXA measurements, improved prediction was not observed[40]. However, this was a preliminary study with only 42 fractures and follow-up work may reveal that differences in fracture prediction between DXA and QCT do in fact exist[40]. Similarly, in the Health, Aging and Body Composition Study, vertebral trabecular vBMD was a significant predictor of non-vertebral fracture, but was not a stronger predictor than hip BMD measured by DXA[34].

2.2 GENETICS OF OSTEOPOROSIS

In addition to demographic characteristics like age, family history is a well-established risk factor for osteoporosis indicating that genetics may play an important role in skeletal health[6]. Additionally, there are many clinical accounts of rare skeletal conditions following a Mendelian inheritance pattern. Expanding from these observations to family-based and population-based studies has confirmed the importance of genetics in osteoporosis and revealed some of the underlying mechanisms of disease.

2.2.1 Heritability

Heritability studies provide much of the evidence that there is a genetic contribution in osteoporosis risk and overall bone health. It is relatively well documented that peak bone mass (as measured by DXA) is highly heritable with estimates from twin and family studies ranging from 40-85%[6-10].

The heritability of bone loss is more controversial. Early on it was noted that heritability was higher at younger ages. Twin studies showed a higher heritability in younger twins[7] and a family study that investigated heritability across the continuum of age determined that the highest heritability was estimated at age 28[8]. This furthered the evidence of heritability of peak bone mass and one could hypothesize that environmental factors became more influential with advancing age. To assess this, a few studies have examined bone loss longitudinally but findings have not been consistent[6, 41-45]. One study of peri- and post-menopausal women saw no evidence that annualized percent BMD loss was heritable at the hip, but did estimate that annualized percent BMD loss at the lumbar spine, whole body and forearm was about 40%[43].

Although there is evidence of heritability of bone loss it is not as striking as studies assessing peak bone mass and there is indication within these studies that environmental factors are also important[6, 45].

Similarly, assessment of the heritability of fracture risk has been inconsistent. Studies have shown that wrist fracture is heritable with estimates ranging from 25-54% [46-48], while other studies have shown no substantial genetic component to osteoporotic fracture[49]. A large twin study from the Swedish Inpatient Registry reported that the heritability of any fracture was only about 16%, whereas the heritability of osteoporotic fracture and hip fracture were substantially higher (27% and 48% respectively)[48]. Furthermore, heritability of all three fracture types were substantially higher for those under 69 years of age compared to older age groups[48]. Although there are some shared genetic components, BMD and fracture may be influenced by different genetic factors[46]. Further complicating the interpretation of these data, it is thought that there may be a genetic component to falls and frailty that are involved in fracture risk but distinct from those genes associated with low BMD[50]. Taken together, this indicates that not only are there different genetic factors regulating BMD and fracture risk, but that environment and possibly gene-environment interactions become more important in assessing the heritability of fracture risk as people age.

As previously discussed, DXA BMD measures do not fully identify those at risk for fracture, are not perfect measures of bone quality, and are confounded by bone size[38]. Therefore, other methods of assessing bone health have been identified. These other measures of bone quality, which may distinguish different biological properties of bone, have also been assessed for heritability to determine the role of genetics. Quantitative ultrasound (QUS) measures, which have been thought to more adequately represent the material properties of bone

than DXA[37], have been assessed for heritability in several studies. In general, QUS appears to be highly heritable with estimates ranging from 51-82%[51-53]. What is interesting is that although there is some overlap, 47-66% of the genetic variance explained by QUS is unique from the genetic variance that explains DXA measures[52-54]. Furthermore, a high genetic correlation between different types of QUS measures at the same skeletal site (94-99%) has been observed[55]. Quantitative computed tomography (QCT) measures of volumetric BMD (vBMD) are becoming more widely integrated into population studies as they are not confounded by bone size and can examine the cortical and trabecular bone compartments separately. However, little is known about the heritability of these measures. Two studies, examining vBMD using peripheral quantitative computed tomography (pQCT) of the axial skeleton, determined that heritability of cortical vBMD was between 29-42% and trabecular vBMD was between 59-70%[56, 57]. The observation that there is a larger genetic component for trabecular vBMD compared to cortical vBMD is of particular interest and may prove to be biologically important. However, the clinical utility of pQCT measures is not yet well defined. One study did obtain heritability estimates of vBMD for the lumbar and thoracic spine and found heritability estimates of 71% and 73% respectively for the two spine measures[58]. Although these estimates were substantially higher than the heritability estimates for the hip and spine BMD measured by DXA (43% and 56% respectively), these differences were not statistically significant[58].

Although these studies show the importance of genetics in determining BMD and osteoporosis risk, they also underscore the complex nature of this relationship. Although heritability studies do not directly test this, they indicate that there is an elaborate interplay of genes involved in peak BMD accrual, bone structural and material properties, bone loss and fracture risk that influence an individual's bone health. Therefore, understanding the genetics of

osteoporosis will likely mean understanding the genetic involvement in all of these individual aspects of bone health.

2.2.2 Population-based Genetics Studies

There are a variety of strategies used to study the role of genetics in osteoporosis. Population-based genetic association studies have been the most commonly employed. To date the vast majority of these studies have been candidate gene studies, where a physiologically defined candidate gene is identified and genetic variation within this locus is tested for association with bone phenotypes. A plethora of these studies have been published, but extensive work has been done on only a limited number of candidate genes such as the vitamin D receptor (VDR), collagen type 1 alpha 1 (COL1A1), estrogen receptor alpha (ESR1) and the low-density lipoproteinreceptor–related protein 5 (LRP5).

Original reports identified an association between an intronic SNP in VDR and BMD at the hip and spine in 250 Caucasian twins[59]. However, conflicting results were observed in later studies with either no association detected or with associations between different VDR polymorphisms and BMD[60-64]. The Genetic Markers for Osteoporosis consortium (GENOMOS), a European consortium made up of 9 research teams at the time of the referenced study, published a large scale investigation of the association between VDR markers and BMD in 26,242 individuals[65]. This study found no evidence for association with any of the commonly studied VDR polymorphisms and BMD, but found a modest association between a SNP located at the Cdx2 transcription factor binding site and fracture and there is functional evidence in support of this association[62, 63, 65].

Genetic variation in the COL1A1 gene, which encodes the major protein component of bone, has also been associated with BMD in population-based samples. Specifically, associations between BMD and variants in the proximal promoter and a mutation in the first intron that creates a binding site for the Sp1 transcription factor have been reported[6, 66, 67]. The Sp1 mutation has been widely studied and functional evidence exists that the creation of this transcription factor binding site increases COL1A1 production[6, 68, 69]. This increase in production is thought to cause an imbalance between the type 1 (COL1A1) and type 2 (COL1A2) collagen chains and result in weaker material properties of bone[6, 68, 69]. These findings were further supported by a large-scale investigation involving 20,786 participants from the GENOMOS consortium that found a lower BMD at both the hip and spine in individuals carrying two copies of the Sp1 mutation[70].

Polymorphisms in the LRP5 gene were associated with familial high bone mass and low bone mass (*Osteoporosis-Pseudoglioma Syndrome)*conditions[71, 72]. These findings led to further investigation of LRP5 as a biologic candidate gene and shed light on the importance of the WNT/β-catenin signaling pathway inskeletal development[73-76]. Specifically, the WNT/β-catenin pathway is thought to influence BMD through its control of bone formation by the regulation of osteoblast differentiation, proliferation and apoptosis[74, 75, 77]. Later work also found associations with common LRP5 polymorphisms and BMD in the general population and this finding was also confirmed in 37,534 participants from 18 research teams in the expanded GENOMOS consortium[78-80].

Technological advances have allowed for whole genome association studies to be completed. Though there are a number of methodological limitations to these studies, they do provide a hypothesis free method of testing for genetic associations in population-based studies

that are not afforded by candidate gene studies. A genome-wide study of 2094 women from the United Kingdom was recently completed and promising findings from this sample were replicated in an additional 6463 men and women from a Dutch population-based study, and two additional studies of women from the United Kingdom[81]. This study identified single nucleotide polymorphisms (SNPs) in or near the LRP5 gene and the osteoprotegerin gene (TNFRSF11B) that were highly associated with lumbar spine and femoral neck BMD measured by DXA[81]. Another genome-wide association study of 5861 Icelandic men and women that validated findings in 7925 men and women from Iceland, Denmark and Australia, also identified TNFRSF11B as a promising gene for bone mineral density[82]. Additionally, this study identified the receptor activator of nuclear factor-kB ligand gene (RANKL), the estrogen receptor 1 (ESR1), a region on chromosome 1 and a region near the major histocompatability complex region (6p21) as being associated with bone mineral density. Although these studies are advantageous in that they are hypothesis free, they have thus far identified previously known candidate genes (TNFRSF11B, LRP5, RANKL and ESR1), chromosomal regions previously identified by linkage studies (see Table 1 in next chapter) and explain very little of the genetic variation in BMD despite the high heritability estimates previously reported for BMD[83-87]. Although genome-wide association studies have the potential to yield new insight into the genetic mechanisms of osteoporosis, it is unlikely that this approach alone will be sufficient to unravel the complex involvement of genetic factors. More likely, the genome-wide association approach coupled with other population and family based studies will be needed to fill in the gaps of our knowledge on the genetics of BMD and osteoporosis.

2.3 SKELETAL SITE DIFFERENCES AT THE HIP AND SPINE

Although bone mineral density (BMD) measurements at the hip and spine are highly correlated and predictive of fractures at distal sites, there is heterogeneity between BMD measures at different skeletal sites and as many as 40% of patients fall into discordant diagnostic categories for osteoporosis when assessing BMD at both the femoral neck and lumbar spine[88-91]. Understanding this heterogeneity would not only improve our understanding of skeletal health, but may help refine diagnostic criteria.

2.3.1 Hip and Spine Fractures

The most significant fracture types in osteoporosis are hip fractures and vertebral fractures. Approximately 300,000 hip fractures occur each year, and are associated with excess death, disability and high medical costs[1, 24]. Less commonly diagnosed and often mistakenly regarded as insignificant, vertebral fractures are an important component of osteoporosis morbidity with an estimated 750,000 occurring annually[1]. Though hip fractures are thought to be the most serious fracture type, vertebral fractures are also associated with decreased quality of life, functional impairment, pain and mortality[24, 92].

In general, low BMD is associated with an increased risk of fracture and evidence is building that low BMD at individual skeletal sites may indicate fracture risk specific to that skeletal site [93, 94]. Ex vivo strength, measured by mechanical bending of excised cadaver bones, indicates that DXA measures of BMD predicted 50-60% of the failure load, or the amount of force a bone can withstand without breaking, at the specific skeletal site measured by DXA. In contrast, only 20-35% of failure load at different skeletal sites can be predicted by DXA

measures[95]. Thus, bone strength is determined to some extent by skeletal-site specific characteristics of bone.

2.3.2 Environmental Factors and Participant Characteristics

BMD patterns across age differ for the hip and spine in men[96, 97]. In the MrOS study, a 5-year increase in age was associated with a 2.6% lower areal BMD at the femoral neck but a 7.0% higher areal BMD at the lumbar spine[97]. This is not a gender specific phenomena and the increased BMD in the spine with age is thought to indicate compression fractures and osteophyte formation as opposed to improved strength of the spine with age[96, 97]. There is a paucity of information on the determinants of vBMD, but in MrOS, older age was associated with a decrease in integral vBMD at the femoral neck[98].

Anthropometric characteristics are associated with BMD measurements at the hip and spine. In general, higher weight and BMI are positively correlated with both hip and spine BMD in men[97, 99, 100]. The influence of height is less clear. Although some studies did not observe a correlation between height and BMD, others observed that taller men have lower hip BMD, but higher BMD at the spine[97, 99, 100].

Muscle strength and physical activity levels also impact BMD in men similarly at both skeletal sites. In general, greater muscle strength is correlated with a higher areal BMD at both the hip and spine as is increased levels of physical activity[99-101]. However, there is some evidence of skeletal site specificity. In the Study of Osteoporotic Fractures in Men a 1% higher hip BMD was observed in those with higher levels of physical activity, but no difference in BMD was observed for spine BMD with differing levels of physical activity[97]. Furthermore, there is evidence from studies in women that adding upper body exercise to a lower body

exercise routine increases BMD at both the hip and spine, whereas those only doing lower body exercises only increase BMD at the hip[102].

Other lifestyle factors including smoking, alcohol use and calcium intake have also been examined for associations with BMD in men. In general, lower BMD at the hip and spine is observed in smokers, but this finding is often not statistically significant, and is often explained by other health factors[97, 99, 100]. Alcohol has been found to be associated with slightly higher hip and spine BMD in the MrOS study, but was only associated with increased spine BMD in a study of older Finnish men[97, 99, 103]. Skeletal site differences have been consistently observed in relation to calcium intake and BMD. Men with higher calcium intake have higher hip BMD, but no difference in spine BMD is detected[97, 99, 100]. Free testosterone has been shown to be an independent predictor of hip BMD, but is not associated with spine BMD[104].

2.3.3 Genetic Factors

There is indication that heritability of BMD may be skeletal site-specific. Specifically, heritability assessment of premenopausal women and their children indicated distinct genetic mechanisms regulating the lumbar spine and femoral neck despite a high correlation of these phenotypes(105). Specifically, this studyidentified higher correlation between mothers and offspring at the same skeletal than across skeletal sites(105). Furthermore, genetic correlations between the lumbar spine, femoral neck and whole body indicate that the loci affecting the lumbar spine are more likely to affect total body BMD than are those loci affecting the femoral neck[106].

Several whole genome linkage studies examining BMD have been completed, and eight of these studies that examined both hip and spine BMD in the same set of participants were evaluated to see if similar linkage signals were observed at both skeletal sites (Table 2)[83-85, 107-111]. Although associations between the same chromosomal locations for the same trait

Table 2-1 Summary of Linkage Scores by Chromosomal Locations for Studies Investigating Lumbar Spine and Hip Bone Mineral Density

Study	Chromosome Position	LOD	Skeletal Site (FN=Femoral Neck; LS=Lumbar Spine)
Devoto (2005)	1p36	2.87	FN
	7p15	2.15	LS
Ralston (2005)	1q23-q24	2.00	FN
	3q25	2.43	LS
	4q25	2.22	FN
	7p14	2.28	FN
	10q21	4.42	FN
	16p13	2.52	LS
Peacock (2005)	1q	3.90	LS
	2p	3.70	LS
	2q	2.99	FN
	14p	4.80	LS
	18q	3	FN
	21	2.80	FN
Shen (2004)	7p14	2.64	LS
	11q23	3.13	LS
	12q24	1.5	LS
Wilson (2003)	3p22	2.72	LS
	16p12-q23	2.11	LS
Karasik (2002)	6p21	2.93	FN
	12q24	2.08	LS
Koller (2000)	1q21-23	3.11	LS
	22q12-q13	2.13	LS
Devoto (1998)	1p36	3.51	FN
	2p23-p24	2.07	LS
	4q23-24	2.28	FN

were detected, detecting associations with different skeletal sites in the same chromosomal region was less common. No individual study identified the same region as being associated with both spine and hip BMD, but the lumbar spine and femoral neck were associated with the

1q21-23 region in the Ralston[109] and Koller[107] studies, and the 7p14 region in the Ralston[109] and Shen[110] studies. It is likely not a coincidence that the Ralston study[109] appears in both of these cases. This study was by far the largest, and would have had the highest power to detect significant association signals. The lack of overlapping linkage signals for different skeletal sites adds support to the notion of skeletal site specific genetic regulation. The minimal overlap between QTLs for different BMD measures in this investigation is certainly in line with the heritability studies done to date[105, 106].

A recent meta-analysis of genome-wide linkage studies for BMD, compared associations within discrete regions of the genome (referred to as bins) and different skeletal sites[112]. No individual bins were associated with both femoral neck and lumbar spine BMD, but when the bins were ranked in terms of their significance level for each trait there was a correlation between ranking of lumbar spine bins and femoral neck bins[112]. This further supports the hypothesis that although may also be some genetic factors that act on BMD globally, there may be many site-specific genetic factors.

2.4 COMPARTMENT SPECIFIC DIFFERENCES

There are two compartments of bone, the trabecular (or spongy) bone compartment and the cortical (or compact bone) compartment. The physiology and structure of these two types of bones fundamentally differ and many of the factors that influence the density of these two bone compartments also differ.

2.4.1 Environmental and Participant Characteristics

Different age patterns have been observed in trabecular and cortical bone. In the Study of Osteoporotic Fractures in Men, age was associated with a sharp (22%) decrease in trabecular vBMD across the age range of the study, but there was no association with cortical vBMD at the femoral neck[98, 113]. However, studies of compartment specific vBMD of the peripheral skeleton show age decreases in both trabecular and cortical vBMD but at different rates[57, 114]. Furthermore, a cross-sectional study examining a larger age rangge (20-90 years) showed a significant decrease in both cortical and trabecular vBMD at the femoral neck in men[115]. However, a much larger percent decrease was seen in trabecular vBMD than in cortical vBMD for men (45% vs 13%) in that cross-sectional study[115].

Data from the MrOS Study indicate that there are differences in anthropometric correlates of vBMD in different compartments. Specifically, higher weight was associated with higher vBMD for both compartments in the femoral neck, but weight change since age 25 was only associated with cortical vBMD[113]. A study of correlates of pQCT measures in Afro-Caribbean families identified weight as an important correlate for both compartments, but in different directions, with weight being positively correlated with trabecular vBMD and negatively correlated with cortical vBMD[57]. Though height was not associated with cortical vBMD in the MrOS Study, both height and change in height since age 25 were associated with trabecular vBMD[113]. Similarly, pQCT findings have indicated that height is negatively associated with trabecular vBMD but that there is no association with cortical bone[57].

In a study of younger men, physical activity was associated with higher trabecular vBMD at both the tibia and radius but there was no difference in cortical vBMD at the tibia and a slightly lower cortical vBMD at the radius[116]. In contrast, data from a population of older

men from the MrOS study observed no difference in vBMD from either compartment at the femoral neck[113]. However, better muscle strength, as measured by grip strength, was associated with higher cortical and trabecular vBMD at the radius[114].

Smoking was not associated with vBMD in either compartment at the radius, but was associated with a lower trabecular vBMD at the femoral neck[113, 114]. Another study examining correlates of pQCT indicates that smoking is associated with lower trabecular vBMD but no difference in cortical vBMD at either the radius or tibia [57]. Alcohol use was not associated with trabecular vBMD, but was positively associated with cortical vBMD at the femoral neck [113]. Higher dietary calcium intake was associated with higher trabecular vBMD but was not associated with cortical vBMD at the femoral neck[113].

2.4.2 Genetic Factors

Differences in trabecular and cortical measures have been observed in inbred mouse strains[117, 118]. Specifically, cortical thickness was shown to differ in a variety of mouse strains[118]. Cortical and trabecular microCT measures differed in three strains of mice previously classified as having high, average and low BMD[119]. A linkage study, investigating differences when bones were mechanically loaded in mice, identified some loci that were independently associated with cortical vBMD but not total vBMD at the same skeletal site[117]. However, this study did not explicitly examine trabecular vBMD[117]. Other studies investigating trabecular traits in mice identified linkage peaks that were distinct from total BMD at any skeletal site[120].

Cortical and trabecular vBMD are highly heritable but there may be a lower heritability for cortical vBMD (17-42%) than trabecular vBMD (59-73%)[56, 57, 121]. A study by Wang et al., of Afro-Caribbean families, examined the genetic correlation between compartment and

skeletal site specific pQCT measures at the radius and tibia[121]. This study found a high correlation within compartments at different skeletal sites (genetic correlation with trabecular vBMD measures was 0.87 and with cortical vBMD measures was 0.83) but not across compartments within the same skeletal site nor across compartments and across skeletal sites (genetic correlation ranged from 0.25-0.32)[121]. Collectively, the evidence from mouse and human studies indicates that although there may be some overlap, distinct sets of genes may influence cortical and trabecular vBMD.

2.5 GENE-ENVIRONMENT INTERACTIONS

Genes function in the context of environments and it is important to consider the influence of environmental factors in the study of complex genetic traits like BMD[122]. Gene-environment interaction occurs when genotypic and environmental effects differ depending on the environment a genotype is in[123]. Gene-environment interactions may be an important avenue of research for prevention and treatment in the anticipated era of personalized medicine[124].

Examples of gene-environment interactions exist for bone mineral density[125, 126]. Interactions between taking hormone replacement therapy and genetic variation in both the interleukin 6 gene (IL6) and the estrogen receptor 1 gene (ESR1) are associated with BMD[127-129]. Genetic variation in IL6 was also associated with hip BMD in those receiving insufficient dietary calcium, but not in those with adequate calcium[128]. Low birth weight, a marker of poor gestational nutrition, may interact with vitamin D receptor (VDR) genotype to influence BMD at the lumbar spine[20]. Although investigating gene-environment interactions is methodologically complex, requires large samples sizes to achieve sufficient statistical power

and involves carefully characterization of environmental factors of interest, these studies are worthwhile because of their potential to identify important biological relationships in osteoporosis.

2.6 GENE-GENE INTERACTIONS

In both human and animal genome-wide linkage and association studies, the main effects identified fail to account for the amount of variation in bone related traits that we would expect to explain based on the findings from heritability studies. However, a more thorough understanding of the genetics of complex traits such as BMD will also likely require insight into how genetic variants interact with each other to influence the variation in a trait. In other words, having the presence of two or more genetic variants associated with low BMD may not result in a lower BMD that is reflective of an additive effect of these two variants. Knowledge of gene-gene or epistatic interactions may not only be important in explaining the variation in BMD, but increased knowledge of these interactions may shed light on the underlying biology that impacts skeletal health.

2.6.1 Animal Studies

Studies of inbred mouse populations are often used to examine epistatic effects because their breeding can be so tightly controlled and the genotypes of the parental generation are known. A genome-wide linkage study of mice from an F2 cross between the inbred strains MRL/MpJ and SJL/J identified several significant gene-gene interactions for both femoral breaking strength and

femoral BMD[130]. These interactions explained a substantial amount of the variation in the F2 generation with 14.6% of the variance observed for femoral breaking strength being explained by 3 locus-locus interactions, and 17.8% of the variation observed for femoral BMD being explained by 2 locus-locus interactions[130]. Another study that crossed Fischer 344 and Lewis F2 rats identified several locus-locus interactions for a variety of skeletal sites and both volumetric and areal BMD[131]. As much as 17% of the phenotypic variation at a given skeletal site was explained by significant epistatic interactions in this study[131]. However, not all studies indicate that there are significant epistatic interactions for BMD. One investigation that crossed C57BL/6J and C3H/H3J mice, which differ in femoral and lumbar spine BMD by 51% and 9% respectively, found virtually no evidence of gene-gene interactions in the progeny and concluded that the loci contributing to BMD are largely independent[132].

2.6.2 Human Population and Family-based Studies

Though often more complicated and costly, investigations of gene-gene interactions are not limited to animal studies. One example is a genome-wide linkage analysis of 451 families having at least one member with low BMD[133]. In this investigation, loci strongly or moderately associated from main-effects analyses were used to test for epistatic interactions and though no significant interactions for hip BMD were observed, two locus-locus interactions for spine BMD were identified[133]. These investigators also completed a study of 20 candidate genes in which a significant interaction between three pairs of genes was observed[134]. The authors note that one pair of genes, the glucocorticoid receptor (GCR) and the estrogen receptor 2 (ESR2), have been shown to biologically interact and counteract each other in their regulation of bone metabolism[134].

The two studies above were completed as parts of larger association and linkage studies and focused on significant main effects to guide the inclusion of genes in the interaction analysis. Another approach to assessing gene-gene interactions would be to identify a set of genes that are thought to interact biologically based on molecular biology investigations, and then interrogate these findings for interactions regardless of whether or not they had important main effects. One example of this approach was a study of 434 families which sought to determine if genetic variants in the COL1A1 gene, an important candidate gene for BMD that makes up the major protein component of bone, interacted with 11 genes thought to regulate osteoclast differentiation[135]. Although this study identified several loci that were significantly associated with BMD at the femoral neck without interacting with other genes, IL6 and TNFRSF1B were only significant when epistatic effects with the COL1A1 gene were taken into account[135].

Altogether these studies demonstrate the importance of genetics in determining BMD, but underscore the complexity of this relationship. Building from these studies we hope to explore the genetic factors associated with volumetric BMD in older Caucasian men.

3.0 ARTICLE 1: A HIGH-DENSITY ASSOCIATION STUDY OF 383 CANDIDATE GENES FOR VOLUMETRIC BONE DENSITY AT THE FEMORAL NECK AND LUMBAR SPINE AMONG OLDER MEN

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3.1 ABSTRACT

Genetics is a well established but poorly understood determinant of bone mineral density (BMD). While some genetic variants may influence BMD throughout the body, others may be skeletal site specific. We initially screened for associations between 4608 tagging and potentially functional single nucleotide polymorphisms (SNPs) in 383 candidate genes and femoral neck and lumbar spine volumetric BMD (vBMD) measured from quantitative computed tomography scans among 862 community-dwelling Caucasian men aged ≥65 years in the Osteoporotic Fractures in Men Study (MrOS). The most promising SNP associations ($P<0.01$) were validated by genotyping an additional 1156 Caucasian men from MrOS. This analysis identified 8 SNPs in 6 genes (DMP1, APC, HOXA, PTN, FGFR2 and FLT1) that were associated with femoral neck vBMD and 13 SNPs in 7 genes (APC, BMPR1B, FOXC2, HOXA, IGFBP2, NFATC1, SOST) that were associated with lumbar spine vBMD in both genotyping samples ($p<0.05$). Although most associations were specific to one skeletal site, SNPs in the APC and HOXA gene regions were associated with both femoral neck and lumbar spine BMD. The current analysis identifies several novel and robust genetic associations for volumetric BMD and these findings in combination with other data suggests the presence of genetic loci for volumetric BMD that are at least to some extent skeletal-site specific.

3.2 INTRODUCTION

Osteoporosis, a condition marked by low bone mineral density (BMD) and an increased risk of fracture, is a significant health burden in older individuals(1). Hip and vertebral fractures are major osteoporotic fractures and occur in approximately 300,000 and 750,000 individuals, respectively each year(2,3). Although osteoporosis is more common in women, it is also a substantial problem among older men. In contrast to our understanding of osteoporosis in women, considerably less is known about the etiology and prevention of osteoporosis in men.

Genetic factors have an established influence on BMD, with heritability estimates indicating that as much as 85% of the population variance in BMD is due to genetic variants (4) and that some of these variants may be skeletal site and sex specific(5). Identification of individual genetic variants by candidate gene association studies has had some success, but many studies have been limited because they characterized a single candidate gene at a time, did not comprehensively investigate genetic variation for the candidate gene of interest and did not validate findings in an independent sample. Further complicating the search for genetic factors contributing to BMD, many studies have had small sample sizes and/or had potential design flaws, such as failing to account for population stratification. Recently, genome-wide association studies have been completed to identify genetic variants that influence dual energy X-ray absorptiometry (DXA) measures of areal BMD(6,7). Although these genome-wide studies have identified several genetic loci of interest, the loci identified explain very little of the variation in BMD(8-12). Thus, much of the genetic variation in BMD remains to be explained. Further, the majority of genetic investigations have measured areal BMD by DXA which is confounded by bone size and may be increased erroneously at the lumbar spine by spinal degenerative disease and aortic calcification(13-17). Volumetric BMD (vBMD) measured by

quantitative computed tomography (QCT), avoids some of the limitations of DXA, but to date, little is known about the genetic determinants of vBMD.

In the current study, we assessed and subsequently validated the associations between common genetic variation in 383 biological candidate genes and vBMD of the femoral neck and lumbar spine among 2,018 older Caucasian men in the Osteoporotic Fractures in Men Study (MrOS).

3.3 METHODS

3.3.1 Study Participants

Participants for this investigation were selected from the Osteoporotic Fractures in Men Study (MrOS). MrOS is a prospective, cohort study designed to investigate anthropometric, lifestyle and medical factors related to bone health in older, community dwelling men. At study entry, participants were at least 65 years old, community-dwelling, ambulatory and had not had bilateral hip replacement(18). In total, 5995 men were recruited from March 2000 through April 2002 primarily using population-based mailings in 6 geographic regions in the United States: Birmingham, AL; Minneapolis, MN; Palo Alto, CA; Pittsburgh, PA; Portland, OR and San Diego ,CA(19).

Caucasian participants with volumetric bone mineral density (vBMD) were selected for genotyping in the current investigation if they had not reported taking bone altering medications such as androgens, anti-androgens or oral corticosteroids and had not reported being on

osteoporosis treatment. Genotyping was completed in two phases using two independent samples from the MrOS cohort: a discovery sample and a validation sample. Specifically, the discovery sample was comprised of 862 Caucasian men with lumbar spine or femoral neck vBMD measures. These men were selected without regard to their BMD level from the Minneapolis and Pittsburgh clinic sites for genotyping. Promising SNPs identified in the discovery sample were then tested for replication in 1156 additional men with vBMD measures in a validation sample that was comprised of men from the remainder of the MrOS clinic sites (Birmingham, Palo Alto, Portland and San Diego).

3.3.2 Volumetric BMD

Volumetric BMD (vBMD) was measured using quantitative computed tomography (QCT) of the hip and lumbar spine. Due to cost restraints, only the first 65% of the MrOS cohort and all non-white participants were referred for QCT scans. There were few differences between the men who did and did not receive QCT scans except that those with QCT scans were slightly younger and more likely to be from a minority population(20).

QCT measurement of the lumbar spine was obtained using an anatomical region 5mm above the L1 superior endplate to 5mm below the L2 inferior endplate and hip scans were obtained in the anatomical region defined by the femoral head to 3.5 cm below the lesser trochanter. Lumbar spine images were acquired using settings of 120 kVp, 150 mA, 1-mm slice thickness and 512x512 matrices. For this the region of interest was the second lumbar vertebra excluding the transverse processes. Hip images were acquired at settings of 80 kVp, 280 mA, 3-mm slice thickness, and 512 x 512 matrix in spiral reconstruction mode. The femoral neck region was defined as the region from the minimum cross-sectional area to the point 25%

towards the maximum cross-sectional area where cross-sectional area was measured along the neutral axis. Different scanners were used at different clinic sites. Specifically, the images were acquired on a GE Prospeed at the Birmingham clinic, a GE Hispeed Advantage at the Minneapolis clinic, a Philips MX-8000 at the Palo Alto clinic, a Siemans Somatom +4 at the Pittsburgh clinic, either a Phillips CT-Twin or Toshiba Acquilion at the Portland site, and a Picker PQ-5000 in San Diego.

QCT images were processed (by TFL) at the University of California at San Francisco using a standardized protocol. Each participant scan included a calibration standard of three hydroxyapatite concentrations (150, 75 and 0 mg/cm3) and these were used to convert between Hounsfield units and vBMD. Differences between the clinic sites exist and are statistically adjusted for in all analyses.

3.3.3 Other baseline characteristics

Participant characteristics including age, race/ethnicity, health history and medication use were obtained by a self-administered questionnaire. Physical characteristics were obtained by clinic staff. Height was measured by Harpenden stadiometer and weight was measured by balance beam scale.

3.3.4 Candidate Gene and Single Nucleotide Polymorphism (SNP) Selection

Physiologically defined candidate genes were identified from publicly available resources. Specifically, literature searches were conducted using Pubmed, evidence of gene expression in normal human trabecular bone cells was obtained from the Skeletal Gene Database

(sgd.nia.nih.gov, no longer available) and the NCBI UniGene database (www.ncbi.nlm.nih.gov/sites/entrez?db=unigene), genes with functions of interest (e.g., "regulation of bone mineralization" or "skeletal development") were obtained from Entrez Gene (www.ncbi.nlm.nih.gov/sites/entrez?db=gene) and Amigo (amigo.geneontology.org/cgi-bin/amigo/go.cgi), genes with a skeletal phenotype in mice were identified from the Jackson Laboratory Mouse Genome Informatics database (http://www.informatics.jax.org/) and the Online Mendelian Inheritance in Man database (www.ncbi.nlm.nih.gov/omim/) was queried for evidence of genes implicated in skeletal conditions in humans. In total, 383 candidate genes were identified for genotyping (Table 3-1).

Publicly available databases were then interrogated for SNP variation in the region surrounding the candidate gene. For the first phase of genotyping (the discovery sample) two SNP selection strategies were utilized. In the first strategy, genetic variation in the region spanning 30kb upstream and 10kb downstream of each candidate gene was captured by creating a reference SNP panel of variants with a minor allele frequency (MAF) of at least 5% in Phase I of the International HapMap Project (www.hapmap.org)(21). Tag SNPs were then selected using a pair-wise correlation method ($r2 \geq 0.80$)(22). Candidate genes that were clustered near each other on the chromosome were tagged as a unit spanning all loci of interest. For example, IGFBP2 and IGFBP5 are located only 7.6kb from each other on chromosome 2. Since the region of interest for these two candidates overlapped they were tagged as a unit. In the second strategy, potentially functional SNPs that were either non-synonymous coding variants, predicted to alter a putative transcription factor binding site in the promoter region, or a putative exon splice enhancer with MAF $\geq 1\%$ were selected for genotyping using the PupaSNP

(pupasuite.bioinfo.cipf.es/) and Promolign (polly.wustl.edu/promolign/main.html) databases(23,24).

Genotyping for the second phase of the project was conducted in a validation sample for promising SNP associations identified from the discovery sample. Specifically, SNPs with a p-value ≤0.015 for either femoral neck or lumbar spine vBMD in the discovery sample were genotyped in the validation sample. Additionally, SNPs with a p-value ≤0.05 in a gene that also had a SNP with p-value ≤0.015 in the discovery sample were included in the second phase of genotyping.

3.3.5 Genotyping

Genomic DNA from frozen whole blood specimens was extracted using the Flexigene protocol (Qiagen; Valencia, CA). Genotyping was completed using the Illumina Golden Gate custom assay. For the discovery sample, 37 participant samples were run as blind duplicates and 4 internal controls were included per plate to ensure reproducibility. We observed 100% reproducibility among the internal controls and 99.9% reproducibility among replicate participant samples. In the validation sample, 26 participant samples and 4 internal controls per plate were included for quality control. We observed 99.9% reproducibility among internal controls and among duplicate participant samples. To ensure maximum genotyping completeness in the validation sample, loci of interest that could not be genotyped using the Illumina Golden Gate assay were genotyped using one of two platforms: the TaqMan allelic discrimination assay system (Applied Biosystems, Foster City, CA) on a 7900HT Real-time PCR instrument with probes and reagents purchased from Applied Biosystems or Sequenom MassARRAY iPLEX Gold technology (Sequenom, Inc; San Diego, CA) with PCR primers

purchased from Invitrogen (Carlsbad, CA). Participant samples were run in duplicate for these platforms and an average reproducibility of 99.8% and 99.9% was observed for TaqMan and Sequenom, respectively.

Loci with a minor allele frequency less than 1% in the genotyping sample (N=129), that did not conform to the expectations of Hardy-Weinberg equilibrium (P<0.0005; N=123) or that had a low call rate (<85%; N=241) were excluded from statistical analysis. Individual samples with a low call rate (<85%) (N=14) or that were highly correlated with another sample (indicating relatedness; n=13) were excluded from the analysis.

3.3.6 Statistical analysis

Although only Caucasian individuals were investigated in this study, population stratification is a potential concern in large-scale genomic analyses(25). Stratification was initially assessed using the program Structure. Structure is a model-based clustering program that parses the participants into sub-populations and assesses if there are distinct populations or admixed individuals(26). We found little evidence of population stratification in the discovery, validation or pooled samples. Nevertheless, we accounted for potential fine scale population sub-structure by employing a principal components method of analysis using uncorrelated SNPs (r<0.2) to calculate the principal components (27).

Analyses of association between genotype and vBMD assumed both an additive and recessive model of inheritance. Linear regression was used to test for an additive association between the number of copies of the minor allele and vBMD. For the recessive model, regression methods were implemented to determine if individuals having two copies of the minor allele differed from those with the other two genotypes. SNPs with 10 or less individuals having

the rare genotype were not tested for the recessive model to minimize spurious findings based on small genotype specific sample sizes. All analyses adjusted for patient age and clinic site in addition to the first principal component of the population sub-structure analysis. SNPs with associations ($p<0.05$) in both the discovery and validation sample and that also had the same direction of association (regression coefficient + or - for both genotyping samples) were considered to be replicated. Therefore, although we did not use a strict Bonferoni p-value to adjust for multiple-testing in the discovery sample, we conservatively required that the same SNP had an equivalent, significant ($p<0.05$) effect in the validation sample to be considered a replication.

Replicated SNP associations were examined further in the pooled sample of 2018 individuals from the discovery and validation samples. In addition to the analyses described above, further adjustment for height and weight was conducted in the pooled sample to determine if body size attenuated the relationship between genotype and vBMD. Linear regression analysis was used to determine the amount of phenotypic variation explained by all of the significant (replicated) SNPs. Since SNPs in the same gene region are often correlated, the colinearity of individual SNPs in the model was assessed. One pair of SNPs in the femoral neck analysis and three in the lumbar spine analysis were highly correlated and in those instances the SNP with the most missing genotypes was dropped from the regression modeling of all significant SNPs.

3.4 RESULTS

The average age of men in the pooled analysis was 74 years (range, 65 –100 years). The men in the discovery and validation samples were similar in age. Men in the validation sample had lower body weight, lower BMI, taller stature and had slightly lower lumbar spine and femoral neck vBMD ($p<0.001$ for all) (Table 3-2).

In the discovery sample, 4108 of 4608 attempted SNPs passed quality control criteria and were analyzed for their association with lumbar spine and femoral neck vBMD. The mean SNP density for candidate genes was 1 SNP per 13.2 kb (range; 1 SNP/3.2 kb – 1 SNP/97 kb). Tag SNPs were selected based on Phase I of the International HapMap Project. Nevertheless, the SNPs included in our analysis tagged on average 64% (range per gene, 1%-100%) of the SNPs with a MAF >5% in phase II of the International HapMap project. Of the SNPs captured by our tag SNP set, the mean max r2 was 0.97.

193 SNPs in 56 genes were associated with femoral neck vBMD and 173 SNPs in 59 genes were associated with lumbar spine vBMD in the discovery sample and were subsequently genotyped in the validation sample (Figure 3-1a and 3-2a). For the femoral neck, there were several SNPs that had significant p-values in both the discovery and validation samples, but the direction of association was not the same in the two samples. Specifically, this occurred for one SNP in PAX3 (rs1367408), IRAK2 (rs779905), EGFR (rs2075109), CYP17A1 (rs12219246), IGF1R (rs3784606) and BMP7 (rs6127983) and for two SNPs in CDK6 (rs2374589 and rs3802073) (Figure 3-1b). 8 SNPs in 6 genes (DMP1, APC, HOXA, PTN, FGFR2 and FLT1) were associated with femoral neck vBMD in both the discovery and validation sample and the association was in the same direction (Table 3-3). The strongest SNP association with femoral neck vBMD in the pooled analysis was with rs4705573 in APC ($p=0.001$). Men who were

homozygous for the minor allele (GG genotype) of this SNP had a 3.4% lower vBMD then men homozygous for the major allele (AA genotype). Although rs1381632 in DMP1 was significant in both the discovery and validation sample, different genetic models were significant and consequently the pooled analysis was not significant (p=0.0961). Additional adjustment for weight and height did not attenuate any of the SNP associations for femoral neck vBMD (Table 3-3). Each SNP only explained a small amount of the variation in femoral neck vBMD in the pooled sample (0.1%-0.5%; see Table 3-3). The two SNPs in APC (rs6594646 and rs4705573) were in linkage disequilibrium (LD) (r2=0.979 and D'=0.991) and rs6594646 was consequently dropped from the regression modeling. The 7 replicated SNPs in APC, DMP1, FGFR2, FLT1, HOXA and PTN included in the regression modeling explained 1.7% of the variation in femoral neck vBMD after accounting for age, clinic, population sub-structure, height and weight.

Four SNPs in four genes (GHSR, rs558572; SOX6, rs1354329; LRP6, rs4477532; TBX3, rs6489968) had significant associations with lumbar spine vBMD in both the discovery and validation sample but the direction of the association was in different directions (Figure 3-2b). For the lumbar spine, there were 13 SNPs in 7 genes (APC, BMPR1B, FOXC2, HOXA, IGFBP2, NFATC1, SOST) had a consistent direction of association in both genotyping samples (Table 3-4). Each individual SNP explained 0.03%-0.89% of the variation in lumbar spine vBMD in the pooled sample. The SNP explaining the most variation in lumbar spine vBMD was rs1877632 in the SOST gene region that explained 0.89% of the phenotypic variation in vBMD. Men with the less common AA genotype for rs1877632 had 6% higher lumbar spine vBMD than men with the more common GG and GA genotypes. As in the femoral neck analysis, rs6594646 and rs4705573 were in LD and rs6594646 was consequently dropped from the regression modeling of significant SNPs for lumbar spine vBMD. In addition, two SNPs in HOXA

(rs6951180 and rs6964896; r2=0.939 and D'=1.000) and two SNPs in BMPR1B (rs3796443 and rs1434536; r2=1.000 and D'=0.992) were in high LD and thus rs6964896 and rs1434536 were dropped from the regression modeling. Collectively, the 10 replicated SNP associations in APC, BMPR1B, FOXC2, HOXA, IGFBP2, NFATC1, and SOST explained 3.5% of the variation in lumbar spine vBMD after accounting for age, clinic, population sub-structure, height and weight.

3.5 DISCUSSION

Though highly heritable, little is known about the genetic variants contributing to BMD variation in men and volumetric BMD in general(28,29). The current study used a two-staged genotyping strategy to investigate the association between SNPs in 383 biologically defined candidate genes and volumetric BMD at the lumbar spine and femoral neck in a large sample of older men. We identified robust associations between SNPs in 11 genes and lumbar spine and femoral neck vBMD and these associations were validated in a separate sample of older men. To our knowledge, associations between SNPs in APC, BMPR1B, DMP1, FLT1, HOXA, IGFBP2, NFATC1, and PTN and BMD have not yet been described. We also confirmed a previously identified association between several SNPs in the sclerostin (SOST), and forkhead box C2 (FOXC2) genes with vBMD (30-34). Importantly, although two of the gene associations were shared between the femoral neck and lumbar spine, the majority were distinct for the femoral neck or lumbar spine. These observations underscore the importance of measuring BMD at multiple skeletal sites in studies aimed at identifying osteoporosis susceptibility genes.

Although most of the candidate gene associations identified were specific to either the femoral neck or lumbar spine, SNPs in the gene encoding adenomatous polyposis coli (APC)

were associated with vBMD at both skeletal sites. The minor alleles of both rs4705573 in the 5' flanking region and rs6594646 in intron 1 of APC were associated with lower vBMD at both the femoral neck and lumbar spine. These SNPs are not known or predicted to influence APC function or expression and may be in linkage disequilibrium with the causal variation. More commonly known for its role in cancer biology, APC targets β-Catenin for degradation in the WNT signaling pathway, an important pathway in bone metabolism(35). APC is expressed in osteoblasts and osteoclasts from adult human bone(36) and mice with osteoblast specific APC deletions have increased bone deposition to such a degree that the marrow space is reduced(37). A third SNP, rs459552, is a non-synonymous variant that is only associated with femoral neck vBMD in this study. Located at codon 1822, this SNP converts an asparagine residue to a valine residue and has been identified in studies of familial adenomatous polyposis(38). In addition to replacing a hydrophilic amino acid with a hydrophobic one, this SNP falls in the B-catenin down regulation domain of the protein and is potentially functional(38,39).

Two SNPS upstream of the homebox (HOXA) gene cluster in the 5' flanking region of HOXA13 were also associated with vBMD at the femoral neck and lumbar spine. The minor allele of rs6951180 was associated with higher BMD at both the femoral neck and lumbar spine and the minor allele of rs6964896 was associated with higher vBMD at the lumbar spine. The HOXA genes are sufficiently close to enable enhancer sharing and it is possible that SNPs in the 5' region of the gene cluster may directly or indirectly influence HOXA gene expression. HOX genes encode a family of transcription factors best known for their role in body patterning during embryonic development. Humans have 39 HOX genes organized into four clusters termed HOX A, B, C and D. The HOXA genes are involved in the normal development of the axial skeleton and limbs(40). The role of HOXA genes in determining BMD is less well characterized, but

HOXA10 contributes to osteoblastogenesis by regulating target genes for osteoblast differentiation and bone formation including RUNX2, alkaline phophatase, bone sialoprotein, and osteocalcin(41). Thus, at least HOXA10 has a role in promoting bone formation beyond its role in patterning the embryonic skeleton. Activation of HOXA genes has also been described during fracture repair and a re-examination of HOXA gene function in adult bone and BMD regulation may be warranted(42).

The four genes that were uniquely associated with femoral neck vBMD in this study are all plausible biological candidates. DMP1 encodes dentin matrix protein 1, an extracellular matrix protein that regulates osteoblast gene expression and is critical for proper mineralization of bone matrix(43). DMP1 null mice have impaired bone mineralization and mutations in DMP1 are known to cause autosomal recessive hypophosphatemia, a disease that manifests as rickets and osteomalacia(44-46). There is also evidence for a BMD quantitative trait locus (QTL) in mice (Bmd2) that encompasses the DMP1 gene(47,48). FGFR2 encodes a transmembrane receptor for fibroblast growth factor which is involved in bone growth and development. Activating and dominant negative mutations in FGFR2 are associated with altered bone mineralization and familial cranial synostosis syndromes(49,50). FLT1 encodes the cell-surface receptor for vascular endothelial growth factor that is involved in osteoclastogenesis and osteoblast differentiation(51-55). FLT1 null mice have decreased bone mineral apposition and bone formation rates and lower trabecular bone volume(56). Pleiotrophin (PTN), also called osteoblast-stimulating factor 1, is an extracellular matrix protein released by osteoblasts that recruits, promotes adhesion of and increases proliferation of osteoprogenitor cells(57,58). PTN transgenic mice have greater bone calcium content by bone volume compared to controls(59).

The five gene associations specific to vBMD at the lumbar spine are also plausible biological candidates. Bone morphogenetic protein receptor, type IB (BMPR1B) encodes a receptor for BMPs, which are involved in osteoblast commitment and differentiation(60). Transgenic mice with a truncated form of BMPR1B display reduced bone formation rates and BMD(60,61). Evidence for a BMD QTL that contains the BMP1RB gene has been identified in mice(48). FOXC2 encodes forkhead box C2, a member of the forkhead/winged helix transcription factor family that serves as a key regulator of embryogenesis. Mutant mice null for FOXC2 show defects in axial skeletogenesis(62). A modest association between a promoter SNP in FOXC2 and vBMD of the radius was reported in a study of Japanese men and women(30). IGFBP2, which encodes an insulin like growth factor binding protein, is thought to target IGFs to bone, is associated with long bone growth and over-expression of this gene product results in shorter bones(63,64). Further, male Igfbp2 knockout mice have reduced cortical and trabecular bone due to thinner trabeculae than controls(65). Additionally, levels of circulating IGFBP2 are negatively correlated with BMD in postmenopausal women(66). Nuclear factor of activated T-cells 1 (NFATC1) is a transcription factor induced by tumor necrosis factor superfamily, member 11 (RANKL) that is involved in both osteoclast and osteoblast regulation (67-69). Mutations in SOST have been associated with sclerosteosis and Van Buchem disease and polymorphisms in SOST have been associated with normal variation in BMD (31-33). Both rs851054 and rs851056 are predicted to lie in the promoter region of SOST and rs851054 is predicted to abolish a sex determining region Y (SRY) binding site whereas rs851056 is predicted to change a transcription factor binding site from the TAL1/TCF3 complex to c-MYC or RUNX1 (24,70).

Several significant associations were observed in both the discovery and validation samples, but the direction of the association was different (BMP7, CDK6, CYP17A1, EGFR, GHSR, IGF1R, IRAK2, LRP6, PAX3, SOX6, TBX3). CYP17A1 which encodes 17α-hydroxylase/17,20-lyase, has been previously studied for its association with BMD. Some studies have identified an association between a promoter variant (rs743572) in CYP17A1 and areal BMD, while others have detected no association, an interaction with BMI or an association with bone size but not density(71-75). Although this promoter variant was not genotyped in our study it is in LD with the SNP identified in our investigation (D'=0.915 and r2=0.670). A non-synonymous coding polymorphism in LRP6 (rs2302685), the gene encoding low density lipoprotein receptor-related protein 6, has also been examined for its relationship with areal BMD. One study identified an association with fracture risk and a modest association with vertebral body size in men, but no association with BMD was confirmed in a larger multi-center investigation although a modest association with vertebral fracture was observed in men(76,77). The non-synonymous coding polymorphism was genotyped, but not associated with vBMD in our study, but a more common intronic variant (rs4477532) was statistically significant (but not in a consistent direction) with lumbar spine vBMD in our study. Although there was not consistent evidence for association in our study, these candidate genes may warrant further examination.

We were unable to document an association with SNPs in several widely studied candidate genes (COL1A1, ESR1, LRP5, VDR) and vBMD in the current study. Two recent genome-wide association studies have identified significant associations with variation near the LRP5 and ESR1 genes and areal BMD but did not confirm associations between SNPs in or near COL1A1 and VDR with BMD at a genome-wide level of significance (6,7). There are several

reasons why we may not have been able to replicate these associations. First, our study includes only men, whereas many of the past candidate genes have focused largely on women. In addition, most of these candidate gene investigations have used areal BMD as opposed to the vBMD measures used in our study. Finally, our study had 70% statistical power to detect a SNP association that explained 1% of the variation in BMD in the screening stage at alpha = 0.01. Thus, we cannot exclude the possibility of a weaker association between SNPs in the ESR1, COL1A1, VDR, or LRP5 genes and vBMD at the femoral neck or lumbar spine in older men.

Three of the SNPs associated in both the discovery and validation samples of our study were also genotyped in a recent genome-wide association study, but were not significant with areal BMD at either the hip or spine ($p>0.05$) (7). Although not directly genotyped in our study, other SNPs in the gene regions associated with vBMD in our study (APC, BMPR1B, DMP1, FGFR2, FOXC2, HOXA, NFATC1 and PTN) also showed associations ($p<0.05$) in this genome-wide association study but did not achieve genome-wide significance. Most notably, rs11984297 located just downstream of the HOXA13 gene was associated with both hip ($p=0.0002$) and spine ($p=0.0014$) BMD but this SNP is not in LD with rs6951180 (D'=0.216 and r2=0.015) or rs6964896 (D'=1.000 and r2=0.008) which were genotyped and associated with BMD in our study(7).

The amount of phenotypic variation in vBMD explained by SNPs in the current study was small; 1.7% for the femoral neck and 3.5% for the lumbar spine. Although small, the magnitude of these findings are comparable to recent genome-wide studies of BMD and other quantitative traits like height(6,7,78). For example, in a recent genome-wide association study of areal BMD, 0.6% and 0.2% of the phenotypic variation in lumbar spine and femoral neck BMD was explained by two SNPs in two genes(6). Given the high heritability of BMD, much of the

variation in BMD explained by genetic factors remains to be identified. Future studies not only examining SNP associations, but also investigating insertion deletion mutations, copy number variants, rare variants (<5% MAF) and interactions between genetic factors and between genetic and environmental factors may explain more of the variation in BMD.

Our study has potential limitations. First, our reference SNP panel for tag SNP selection was based on Phase I of the International Haplotype Map Project (HapMap), and consequently does not provide as comprehensive a set of tag SNPs as current projects based on Phase II of HapMap. However, the SNPs included in this analysis tagged an average of 64% of the SNPs with over 5% MAF in Phase II of HapMap with a mean max r2 of 0.97. To put this in perspective, our tag SNP set captured 82% and 95% of the SNPs with MAF over 5% in the HOXA and APC gene regions whereas a recent genome wide study of BMD examining over 300,000 SNPs captured 68% and 74% for these gene regions(7). Our analysis also focused on SNPs with a minor allele frequency (MAF) of ≥5% and potentially functional SNPs with a MAF of ≥1% and thus cannot exclude the possible contributions of less common variants in the candidate genes that were investigated. Our study is also limited to Caucasian men aged 65 years and older and our results may not be generalizable to other ethnic groups or to women. This is a cross-sectional analysis of vBMD in a population of men that are likely losing bone (femoral neck integral vBMD is 9% lower in the oldest compared to the youngest men) and could be confounded by differences in genes influencing peak BMD versus genes influencing bone loss(20). Future studies including other volumetric traits including bone structural geometry and bone loss may give further insight into the genetics of osteoporosis in men. Though some of the associated SNPs in the current study are predicted to be functional, none are known to influence gene expression or function and are likely in linkage disequilibrium with the causal variant(s).

Additional genotyping in the gene regions of interest will be needed in this and in other ethnically diverse populations to refine the association signals and to inform future in vitro functional studies.

Although limited to studying only older Caucasian men, this study provides the first large-scale assessment of the genetic contributions to a unique skeletal trait (QCT volumetric BMD), assessed and adjusted for potential population stratification, identified a number of novel and robust genetic associations by including a two-stage internal replication design and collectively, explained more of the phenotypic variance in BMD than other genetic association studies to date (6). This study identifies novel genetic associations and suggests that distinct genetic factors may contribute to lumbar spine and femoral neck BMD in older men. Additional studies in women and other ethnic populations are needed to confirm and extend the present findings.

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3.7 TABLES AND FIGURES

Table 3-1 Candidate genes screened for association with bone mineral density in the discovery sample.

Chr1	DVL1, TNFRSF1B, PAX7, ALPL, WNT4, ID3, CSF3R, LEPR, TGFBR3, DNTTIP2, CSF1, HSD3B2, HSD3B1, NOTCH2, GNRHR2, CTSK, IL6R, ZBTB7B, BGLAP, MEF2D, NTRK1, RXRG, ADIPOR1, MYOG, HSD11B1, TGFB2, WNT9A, WNT3A
Chr2	ID2, NCOA1, POMC, LTBP1, CYP1B1, LHCGR, PPP3R1, IL1R2, IL1R1, IL1A, IL1B, IL1RN, EN1, GLI2, LCT, NR4A2, TANK, DLX1, DLX2, ATF2, HOXD13, HOXD12, HOXD11, HOXD10, HOXD9, HOXD8, HOXD4, HOXD3, HOXD1, FRZB, MSTN, STAT1, CASP8, FZD7, BMPR2, FZD5, IGFBP2, IGFBP5, WNT10A, IHH, PAX3, IRS1, TWIST2
Chr3	IRAK2, GHRL, PPARG, WNT7A, THRB, TGFBR2, MYD88, ACVR2B, CTNNB1, PTHR1, WNT5A, POU1F1, GSK3B, CASR, GATA2, TRH, SHOX2, GHSR, TNFSF10, CHRD, AHSG, ADIPOQ, OSTN, HES1
Chr4	FGFR3, MSX1, NKX3-2, PPARGC1A, KDR, GNRHR, GC, BMP2K, BMP3, DMP1, IBSP, MEPE, SPP1, BMPR1B, NFKB1, DKK2, LEF1, EGF, FGF2, SMAD1, SFRP2, CASP3
Chr5	LIFR, PTGER4, GHR, FST, IL6ST, MAP3K1, CRHBP, MEF2C, APC, HSD17B4, CSF2, PDLIM4, TCF7, HDAC3, FGF1, NR3C1, CSF1R, SPARC, FGF18, MSX2, PROP1
Chr6	RIPK1, SOX4, TNF, CYP21A2, RXRB, PPARD, MAPK14, CDKN1A, VEGFA, RUNX2, OSTM1, WISP3, ENPP1, CTGF, TNFAIP3, ESR1, IGF2R
Chr7	TWIST1, IL6, GPNMB, HOXA1, HOXA2, HOXA3, HOXA4, HOXA5, HOXA6, HOXA7, HOXA9, HOXA10, HOXA11, HOXA13, CRHR2, GHRHR, SFRP4, GLI3, IGFBP1, IGFBP3, EGFR, FZD1, CDK6, CALCR, DLX6, DLX5, CYP3A4, LEP, SMO, NRF1, PTN, TRPV5, CASP2, SHH
Chr8	EGR3, TNFRSF10A, GNRH1, STAR, FGFR1, SFRP1, IKBKB, DKK4, CRH, NCOA2, HEY1, KLF10, FZD6, EXT1, TNFRSF11B, FBXO32, WISP1, CYP11B1
Chr9	CER1, IFNB1, CNTFR, OSTF1, NTRK2, ROR2, OGN, PTCH1, HSD17B3, TGFBR1, TLR4, TRAF1, WDR5, RXRA, NOTCH1, TRAF2
Chr10	DKK1, EGR2, BMPR1A, CHUK, CYP17A1, FGFR2
Chr11	IGF2, CDKN1C, DKK3, PTH, CALCA, SOX6, MYOD1, BDNF, TRAF6, EXT2, CNTF, ESRRA, LTBP3, FOSL1, TCIRG1, LRP5, CCND1, FGF3, FADD, CHRDL2, ARRB1, WNT11, FZD4
Chr12	WNT5B, ADIPOR2, FGF23, NTF3, TNFRSF1A, LRP6, MGP, SOX5, PTHLH, VDR, WNT10B, WNT1, IGFBP6, SP7, CYP27B1, WIF1, IRAK3, MYF6, MYF5, DCN, IGF1, TBX3, HNF1A, P2RX7
Chr13	FLT1, KL, POSTN, TNFSF11
Chr14	NFKBIA, PAX9, BMP4, ESR2, LTBP2, FOS, TGFB3, TSHR, DLK1, AKT1
Chr15	GREM1, CYP19A1, MAP2K1, SMAD3, CYP11A1, CYP1A1, CYP1A2, NTRK3, IGF1R, MEF2A
Chr16	AXIN1, CLCN7, IGFALS, MAPK3, RBL2, CBFB, TRADD, HSD17B2, FOXC2
Chr17	ALOX15, ARRB2, DVL2, SHBG, PIK3R5, CSF3, THRA, IGFBP4, HSD17B1, SOST, MAP3K14, CRHR1, PHOSPHO1, DLX3, COL1A1, TOB1, NOG, TBX2, TBX4, GH1, SOX9
Chr18	MC2R, SMAD2, SMAD4, TCF4, TNFRSF11A, BCL2, NFATC1
Chr19	KISS1R, MAP2K2, MEF2B, CEBPA, NFKBIB, DLL3, AKT2, TGFB1, FOSB, BAX, LHB, OSCAR
Chr20	GNRH2, BMP2, JAG1, PAX1, ID1, E2F1, GDF5, RBL1, GHRH, SRC, WISP2, MMP9, NCOA3, CEBPB, CYP24A1, BMP7
Chr21	IFNAR2, IFNAR1, RUNX1, ETS2
Chr22	COMT, KREMEN1, LIF, CSF2RB, ATF4, MCHR1, PPARA
X Chr	STS, RPS6KA3, GATA1, AR, BGN, IRAK1, IKBKG

Gene symbols are presented by chromosome in order across the given chromosome.

Table 3-2 Participant characteristics (Mean and Standard Deviation).

	Discovery Sample	Validation Sample	Pooled Sample (Discovery + Validation)
	N=862	N=1156	N=2018
Age	74 (5.8)	74 (6.0)	74 (5.9)
Weight (Kg)	85.3 (14.1)	82.9 (12.5)*	83.9 (13.3)
Height (cm)	173.5 (6.7)	174.9 (6.7)*	174.3 (6.7)
BMI	28.3 (4.1)	27.1 (3.6)*	27.6 (3.9)
Lumbar Spine vBMD (g/cc)	0.243 (0.041)	0.227 (0.038)*	0.234 (0.040)
Femoral Neck vBMD (g/cc)	0.304 (0.056)	0.274 (0.051)*	0.287 (0.056)

***Significant difference between discovery and validation sample ($p<0.001$)**

Table 3-3 Replicated associations for femoral neck volumetric BMD.

				Discovery Sample		Validation Sample		Pooled Sample				
				adjustment 1[b]		adjustment 1[b]		adjustment 1[b]		adjustment 2[c]		
Gene	SNP	Allele	Frequency[a]	*β*	*P-value*	*β*	*P-value*	*β*	*P-value*	*β*	*P-value*	r^{2d}
APC	rs459552	T→A	0.228	0.007	0.044 [add]	0.014	0.024 [rec]	0.005	0.011 [add]	0.005	0.014 [add]	0.003
APC	rs4705573	A→G	0.480	-0.008	0.003 [add]	-0.008	0.022 [rec]	-0.009	0.001 [rec]	-0.009	0.001 [rec]	0.005
APC	rs6594646	A→G	0.497	-0.006	0.021 [add]	-0.008	0.023 [rec]	-0.008	0.003 [rec]	-0.008	0.004 [rec]	0.004
DMP1	rs1381632	A→T	0.228	0.007	0.037 [add]	0.014	0.049 [rec]	0.003	0.096 [add]	0.008	0.122 [rec]	0.001
FLT1	rs1408245	C→G	0.166	-0.032	0.010 [rec]	-0.007	0.014 [add]	-0.021	0.003 [rec]	-0.020	0.004 [rec]	0.004
FGFR2	rs7916940	G→A	0.299	-0.013	0.050 [rec]	-0.005	0.052 [add]	-0.004	0.019 [add]	-0.004	0.018 [add]	0.003
HOXA[e]	rs6951180	A→G	0.133	0.010	0.017 [add]	0.024	0.039 [rec]	0.026	0.008 [rec]	0.024	0.015 [rec]	0.003
PTN	rs322297	A→C	0.116	0.010	0.014 [add]	0.007	0.036 [add]	0.008	0.002 [add]	0.008	0.003 [add]	0.004

Additive and recessive models were tested for each SNP and the regression parameter and p-value from the most significant genetic model (additive ore recessive) is shown.
add: p-value from the additive model; rec: p-value from recessive model
a) Minor allele frequency in the pooled sample
b) Adjustment 1: Age, clinic site, population sub-structure
c) Adjustment 2: Age, clinic site, population sub-structure, height, body weight
d) Amount of variation explained after adjusting for age, site, population sub-structure, height, body weight
e) HOXA genes were tagged as a cluster. They include HOXA1, HOXA2, HOXA3, HOXA4, HOXA5, HOXA6, HOXA7, HOXA9, HOXA10, HOXA11, HOXA13

Table 3-4 Replicated findings for lumbar spine volumetric BMD.

				Discovery Sample		Validation Sample		Pooled Sample				
				adjustment 1[b]		adjustment 1[b]		adjustment 1[b]		adjustment 2[c]		
Gene	SNP	Allele	Frequency[a]	*β*	*P-value*	*β*	*P-value*	*β*	*P-value*	*β*	*P-value*	r^{2d}
APC	rs4705573	A→G	0.477	-0.005	0.007 [add]	-0.005	0.048 [rec]	-0.006	0.005 [rec]	-0.005	0.007 [rec]	0.004
APC	rs6594646	A→G	0.494	-0.004	0.035 [add]	-0.005	0.043 [rec]	-0.005	0.015 [rec]	-0.004	0.023 [rec]	0.003
BMPR1B	rs1434536	A→G	0.443	0.005	0.006 [add]	0.006	0.032 [rec]	0.007	0.001 [rec]	0.007	0.001 [rec]	0.005
BMPR1B	rs3796443	A→G	0.439	0.006	0.005 [add]	0.006	0.017 [rec]	0.008	3.2E-04 [rec]	0.008	3.0E-04 [rec]	0.006
FOXC2	rs3751797	T→A	0.260	-0.016	0.007 [rec]	-0.004	0.029 [add]	-0.004	0.004 [add]	-0.004	0.003 [add]	0.004
HOXA[e]	rs6951180	A→G	0.134	0.009	0.003 [add]	0.025	0.004 [rec]	0.008	3.7E-05 [add]	0.007	2.5E-04 [add]	0.007
HOXA[e]	rs6964896	C→A	0.127	0.008	0.004 [add]	0.006	0.008 [add]	0.007	8.1E-05 [add]	0.006	0.001 [add]	0.006
IGFBP2	rs10932669	C→A	0.134	0.006	0.036 [add]	0.004	0.052 [add]	0.005	0.002 [add]	0.006	0.002 [add]	0.005
NFATC1	rs177820	A→G	0.365	-0.005	0.027 [add]	-0.006	0.052 [rec]	-0.008	0.003 [rec]	-0.007	0.008 [rec]	0.004
SOST	rs1534401	A→G	0.380	-0.004	0.030 [add]	-0.006	0.050 [rec]	-0.006	0.012 [rec]	-0.003	0.012 [add]	0.003
SOST	rs1877632	G→A	0.313	0.008	0.001 [rec]	0.011	0.005 [rec]	0.013	1.5E-05 [rec]	0.006	1.4E-05 [add]	0.009
SOST	rs851054	A→G	0.381	-0.004	0.054 [add]	-0.006	0.046 [rec]	-0.006	0.009 [rec]	-0.006	0.008 [rec]	0.004
SOST	rs851056	C→G	0.380	-0.004	0.054 [add]	-0.006	0.043 [rec]	-0.006	0.008 [rec]	-0.006	0.008 [rec]	0.004

Additive and recessive models were tested for each SNP and the regression parameter and p-value from the most significant genetic model (additive ore recessive) is shown.
add: p-value from the additive model; rec: p-value from recessive model
a) Minor allele frequency in the pooled sample
b) Adjustment 1: Age, clinic site, population sub-structure
c) Adjustment 2: Age, clinic site, population sub-structure, height, body weight
d) Amount of variation explained after adjusting for age, site, population sub-structure, height, body weight
e) HOXA genes were tagged as a cluster. They include HOXA1, HOXA2, HOXA3, HOXA4, HOXA5, HOXA6, HOXA7, HOXA9, HOXA10, HOXA11, HOXA13

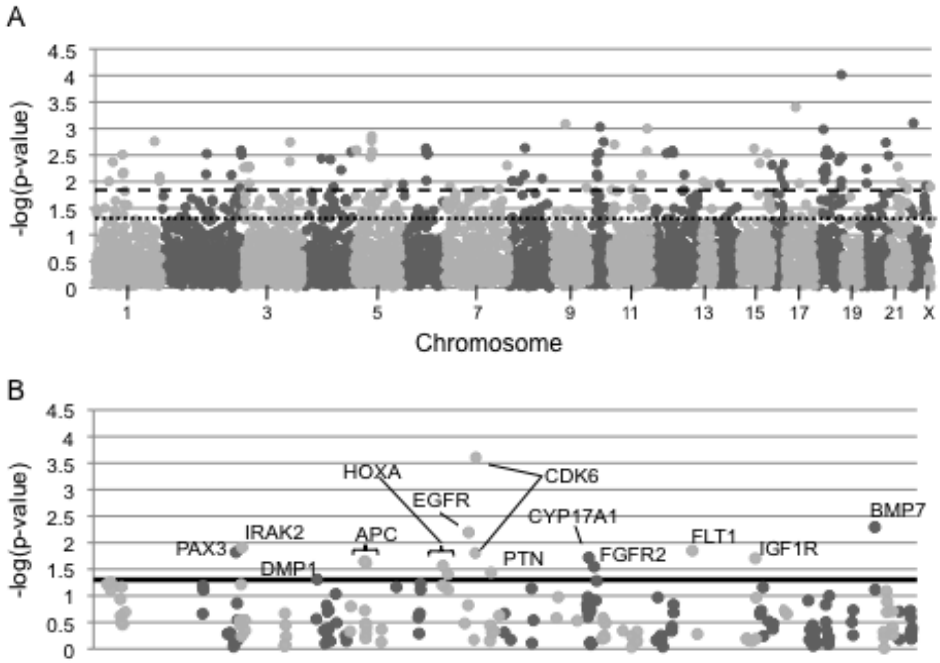

Figure 3-1 SNP association results for femoral neck volumetric bone mineral density.

Association results for the femoral neck are presented for the first phase of genotyping (discovery sample) in *Figure 3-1a* and in the validation sample (*Figure 3-1b*). Specifically, the –log of the p-value observed is presented on the y-axis. The most significant result of the two models tested (either additive or recessive) is presented for each SNP. The SNPs are ordered across the x-axis by chromosome and the base pair position on the chromosome. Odd numbered chromosomes and the X chromosome are presented in light grey. Even numbered chromosomes are presented in dark grey. In *part a* the dark dashed line represents p=0.015 and the dotted line represents p=0.05. The dotted line in *part b* represents p=0.05 and SNPs with p-values ≤0.05 are labeled with the gene symbol that they lie in.

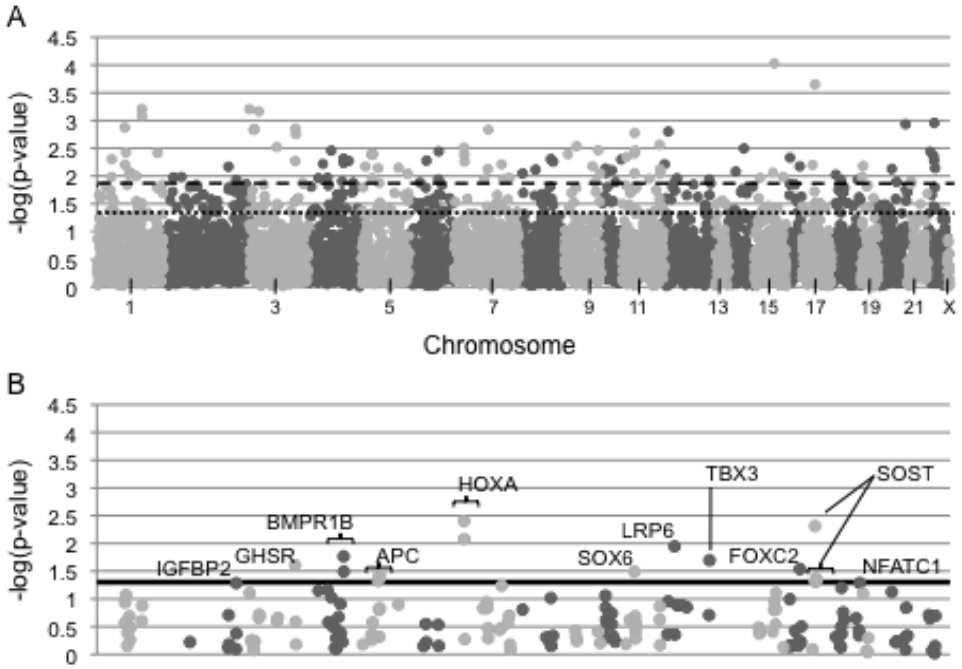

Figure 3-2 SNP association results for lumbar spine volumetric bone mineral density.

Association results for the lumbar spine are presented for the discovery (*Figure 3-2a*) and validation sample (*Figure 3-2b*). Specifically, the –log of the p-value observed is presented on the y-axis and SNPs are ordered across the x-axis by chromosome and base pair position. The most significant result of the two models tested (either additive or recessive) is presented for each SNP. Odd numbered chromosomes and the X chromosome are presented in light grey. Even numbered chromosomes are presented in dark grey. In *Figure 3-2a* the dark dashed line represents p=0.015 and the dotted line represents p=0.05. The dotted line in *Figure 3-2b* represents p=0.05 and SNPs with p-values ≤0.05 are labeled with the gene symbol that they lie in.

4.0 ARTICLE 2: CANDIDATE GENE ANALYSIS OF FEMORAL NECK TRABECULAR AND CORTICAL VOLUMETRIC BONE MINERAL DENSITY IN OLDER MEN

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4.1 ABSTRACT

In contrast to conventional dual-energy X-ray absorptiometry, quantitative computed tomography measures trabecular and cortical volumetric bone mineral density (vBMD) separately. Little is known about the individual genetic variants associated with these two bone compartments in humans, although both may be important for determining bone strength and osteoporotic risk. In the current analysis, we tested the hypothesis that there are different genetic variants associated with trabecular and cortical vBMD at the femoral neck by genotyping 4608 tagging and potentially functional single nucleotide polymorphisms (SNPs) in 383 bone metabolism candidate genes in 822 Caucasian men aged ≥65 years from the Osteoporotic Fractures in Men Study (MrOS). Promising SNP associations were then tested for replication in an additional 1155 men from the same study. We identified SNPs in 5 genes (IFNAR2, NFATC1, SMAD1, HOXA and KLF10) that were robustly associated with cortical vBMD and SNPs in 9 genes (APC, ATF2, BMP3, BMP7, FGF18, FLT1, TGFB3, THRB, and RUNX1) that were associated with trabecular vBMD. Consistent with animal studies, these results suggest that genetic loci for cortical and trabecular volumetric BMD at the femoral neck may be to some extent unique for each bone compartment.

4.2 INTRODUCTION

Bone mineral density (BMD) is an established determinant of bone strength and osteoporotic fracture risk. BMD assessed by conventional dual-energy X-ray absorptiometry (DXA) measures the total bone mineral content in a projected area (integral areal BMD) and cannot directly measure other skeletal features that may also contribute to bone strength, such as the relative amounts of cortical and trabecular bone. In contrast, quantitative computed tomography provides a direct measure of cortical and trabecular volumetric BMD and both of these bone compartments may contribute to bone strength and fracture risk(1,2).

BMD is a highly heritable complex trait that is under context specific genetic regulation(3). For example, the individual genetic factors contributing to BMD variation may differ between women and men, between skeletal sites and between trabecular and cortical bone (4-11). However, the vast majority of studies in humans have searched for genetic variants associated with DXA measures of areal BMD, and most have focused on women. To better understand the genetic determinants of compartment specific volumetric BMD (vBMD) in humans, we explored the association of common genetic variation in 383 bone metabolism candidate genes with cortical and trabecular volumetric BMD (vBMD) of the femoral neck in 1977 men from the Osteoporotic Fractures in Men Study (MrOS).

4.3 METHODS

4.3.1 Genotyping Sample

This study utilized a two-stage genotyping design with two independent samples of older Caucasian men. Both genotyping samples were comprised of men from the Osteoporotic Fractures in Men Study (MrOS). This study has been described extensively elsewhere, but in brief, MrOS is a prospective cohort study of skeletal health in 5,995 community-dwelling men age 65 and older(12,13). Men were recruited into the study from 6 clinical centers across the United States. At the time of entry into the study men were ambulatory and had at least one native hip.

The genotyping samples were comprised of MrOS participants that were Caucasian, had vBMD measurements at the femoral neck, did not report taking bone-altering medications (androgens, anti-androgens or oral corticosteroids) and did not report being on osteoporosis treatment. The first phase of genotyping was carried out on a discovery sample which included 822 Caucasian men with cortical or trabecular vBMD measures at the femoral neck. These men were selected without regard to their BMD level from the Minneapolis and Pittsburgh clinic sites. Promising SNP associations identified in the discovery sample were then tested for replication in a validation sample consisting of 1156 additional men from the four other MrOS clinic sites (Birmingham, Palo Alto, Portland and San Diego).

Participant Characteristics

At the baseline visit, clinic staff obtained participant characteristics including age, medical history and medication by questionnaire. Height was measured by Harpenden

stadiometer and weight was measured by balance beam scale with participants wearing light clothing and no shoes.

4.3.2 Volumetric BMD

Volumetric BMD (vBMD) of the femoral neck was measured using quantitative computed tomography (QCT). To contain costs, only the first 65% of the MrOS cohort and ethnic minorities were referred for QCT scans. Men having had a hip replacement were not eligible for a hip scan. Those who received a QCT scan were similar to those who did not (14).

To measure vBMD at the femoral neck, a QCT scan of the pelvic region (from the femoral head to 3.5 cm below the lesser trochanter) was acquired at settings of 80 kVp, 280 mA, 3-mm slice thickness, and 512 x 512 matrices. Images were acquired using a GE Prospeed (Birmingham), GE Hispeed Advantage (Minneapolis), Philips MX-8000 (Palo Alto), Siemans Somatom +4 (Pittsburgh), Philips CT-Twin(Portland), Toshiba Acquilion (Portland) site, or Picker PQ-5000 (San Diego). Differences between the clinic sites exist and are statistically adjusted for in all analyses.

Each participant scan included a calibration standard of three hydroxyapatite concentrations (150, 75 and 0 mg/cm3). Images were converted from the native scanner Hounsfield Units (HU) to equivalent concentration (g/cm3) of calcium hydroxyapatite contained in the calibration standard. Regions of interest (ROI) in the left proximal femur were identified in QCT images reformatted along the neutral axis of the femoral neck. The periosteal boundary of the femur was determined with a threshold-based region growing algorithm. Using this boundary, the cross-sectional area in each slice along the neutral axis of the femoral neck between the proximal femoral neck and the lateral edge of the trochanter was calculated, and the

minimum and maximum areas were determined. The femoral neck ROI was defined as the portion of the neck extending from the slice with minimum cross-sectional area (medial boundary) to a point 25% of the distance toward the maximal cross-sectional area. Integral volume of the ROI was computed as the total volume within the periosteal boundary. A trabecular volume of the ROI was obtained by applying an erosion process to the integral volume to retain the same shape in a region fully contained within the medullary space. The cortical volume was then defined by applying a threshold of 0.35 g/cm3 to all voxels between the periosteal boundary and the outer boundary of the trabecular volume. All QCT scans were transferred to the University of California at San Francisco for processing and central review. Volumetric BMD for trabecular and cortical compartments was computed over all voxels in the respective volumes. In a group of postmenopausal women, coefficients of variation for the QCT analysis used ranged from 0.6%-3% for vBMD measures(15).

4.3.3 Candidate Gene and Single Nucleotide Polymorphism (SNP) Selection

Candidate genes for vBMD were identified using evidence from several sources including literature searches (Pubmed, www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed), evidence of gene expression in a normal human trabecular bone cells (Skeletal Gene Database, sgd.nia.nih.gov, no longer available; NCBI UniGene, www.ncbi.nlm.nih.gov/sites/entrez?db=unigene), genes with functions of interest such as “regulation of bone mineralization” or “skeletal development” (Entrez Gene, www.ncbi.nlm.nih.gov/sites/entrez?db=gene; Amigo, amigo.geneontology.org/cgi-bin/amigo/go.cgi), genes with a skeletal phenotype (Jackson Laboratory Mouse Genome, http://www.informatics.jax.org) and genes implicated in human

skeletal disorders (Online Mendelian Inheritance in Man, www.ncbi.nlm.nih.gov/omim). From these resources, 383 candidate genes were identified for genotyping(16).

For the first phase of genotyping (discovery sample), publically available data were interrogated for SNP variation in the region surrounding the candidate gene. This was accomplished by first creating a reference SNP panel of variants with a minor allele frequency (MAF) of at least 5% in Phase I of the International HapMap Project (www.hapmap.org) in the genomic region spanning 30kb upstream of the transcription start site and 10kb downstream of the candidate gene transcript (17). Tag SNPs were selected from this reference SNP panel using a pair-wise correlation method ($r2 \geq 0.80$)(18). Candidate genes located near each other on the chromosome were tagged as a unit spanning all loci of interest (such as IGFBP2 and IGFBP5 which are located only 7.6kb from each other on chromosome 2). In addition to tag SNPs, potentially functional SNPs that were non-synonymous coding variants, predicted to alter a putative transcription factor binding site in the promoter region, or a putative exon splice enhancer with MAF ≥1% were selected for genotyping. These putative functional SNPs were selected using either PupaSNP (pupasuite.bioinfo.cipf.es/) or Promolign (polly.wustl.edu/promolign/main.html) (19,20).

In the second phase of the project (the validation sample), promising SNP associations from the discovery sample were replicated. Specifically, SNP associations with a p-value ≤0.015 for either cortical or trabecular vBMD in the discovery sample were then genotyped in the validation sample. Additionally, SNPs with a p-value ≤0.05 in a gene that also had a SNP with p-value ≤0.015 in the discovery sample were genotyped in the validation sample.

4.3.4 Genotyping

Genomic DNA was extracted from frozen whole blood specimens using the Flexigene protocol (Qiagen; Valencia, CA). Genotyping in the discovery sample and the majority of genotyping in the validation sample was completed using the Illumina Golden Gate custom assay. Blind duplicate samples and internal controls were included to ensure reproducibility. For the discovery sample we observed 100% reproducibility among the 4 internal controls run on each plate and 99.9% reproducibility among the 37 duplicate participant samples. In the validation sample, we observed 99.9% reproducibility among the 4 internal controls run on each plate and 99.9% reproducibility among the 26 blind duplicate samples. To ensure maximum genotyping completeness in the validation sample, loci of interest that could not be successfully genotyped using the Illumina Golden Gate assay were genotyped using one of two platforms: the TaqMan allelic discrimination assay system (Applied Biosystems, Foster City, CA) on a 7900HT Real-time PCR instrument with probes and reagents purchased from Applied Biosystems or Sequenom MassARRAY iPLEX Gold technology (Sequenom, Inc; San Diego, CA) with PCR primers purchased from Invitrogen (Carlsbad, CA). Participant samples were run in duplicate for these platforms and an average reproducibility of 99.8% and 99.9% was observed for TaqMan and Sequenom, respectively.

Several participants' samples were dropped from analysis because they had a low call rate (<85%) (N=14) or were highly correlated with another sample indicating relatedness (n=13). Before analysis of the discovery sample, 500 loci were dropped based on pre-defined quality control parameters. Specifically, loci in the discovery sample with an observed minor allele frequency less than 1% (N=129), that did not conform to the expectations of Hardy-Weinberg

equilibrium (P<0.0005; N=123) or that had a call rate (<85%; N=241) were excluded from statistical analysis.

The mean SNP density for candidate genes included in the analysis was 1 SNP per 13 kilobase pairs (kbp) (range: 1 SNP/3 kbp – 1 SNP/97 kbp). Tagging SNPs were selected based on Phase I of the International HapMap Project. Nevertheless, the 4108 SNPs successfully genotyped and included in our analysis (4608 attempted) tagged on average 64% of the SNPs with a MAF >5% in the more recent phase II of the International HapMap project (range per gene: 1%-100%). Of the SNPs captured by our tag SNP set, the mean max r2 was 0.97.

4.3.5 Statistical analysis

Population stratification was initially assessed using the model-based clustering program Structure (21). There was little evidence of population stratification, but we accounted for potential fine scale population sub-structure in subsequent analyses using a principal components method of analysis(22). Uncorrelated SNPs (r<0.2) were used to calculate the principal components for the discovery, validation and pooled samples.

Genetic analyses assumed both an additive and recessive model of inheritance. Specifically, linear regression was used to test for an additive association between the number of copies of the minor allele and vBMD. Regression methods were implemented for the recessive model to test if individuals with two copies of the minor allele differed from those with the other genotypes. For instances where a SNP had fewer than 10 individuals with the rare genotype, only the additive model was tested to minimize spurious findings based on small genotype specific sample sizes. Analyses were adjusted for patient age, clinic site and the first principal component from the population sub-structure analysis. SNPs significantly associated in both the

discovery and validation sample ($p<0.05$) and having an association in the same direction for both genotyping samples (a positive or negative regression coefficient for both samples) were considered replicated findings. Replicated SNP associations were examined further in the pooled sample of 1977 individuals from the discovery and validation samples. In addition to the analyses described above, further adjustment for height and weight was conducted in the pooled sample to determine if body size attenuated the relationship between genotype and vBMD. Linear regression analysis was used to determine the amount of phenotypic variation explained by the significant replicated SNPs. Correlation between individual SNPs in the model was assessed to minimize collinearity in the model. For instances where SNPs in the model were highly correlated, the SNP with the most missing genotypes was dropped from the regression modeling.

4.4 RESULTS

The average age of the 822 men in the discovery sample was 73 years (range 65-100 years) and did not differ from the validation sample (Table 4-1). Participants in the validation sample were slightly taller, weighed less, had a lower BMI and had lower cortical and trabecular vBMD at the femoral neck ($p<0.001$ for all).

Of the 4108 SNPs genotyped in the discovery analysis, 191 SNPs in 72 genes were associated with cortical vBMD and were genotyped in the validation sample (Figure 4-1a). Of these 191 SNPs, 7 SNPs in 5 genes were consistently associated with cortical vBMD in the validation sample (Figure 4-1b). One SNP was identified in the IFNAR2 (rs2834160), NFATC1 (rs177820) and SMAD1 (rs1874572) gene regions while two SNPs were identified in the HOXA

cluster of genes (rs6951180 and rs6964896) and KLF10 gene region (rs3133287 and rs1434278). In addition, one SNP in IGF1R (rs3784606) and one SNP in TCF4 (rs7240986) were significant in both the discovery and validation samples, but the direction of the association was inconsistent.

A pooled analysis was conducted to determine the amount of variation in cortical vBMD that each of the 7 significant SNPs explained (Table 4-2). The most significant SNP association was with rs177820 in the NFATC1 gene region (p=4x10-4) which explained 0.5% of the phenotypic variation in cortical vBMD. For this SNP, men with the less common GG genotype had 1.2% lower cortical vBMD than those with the AA genotype. Additional adjustment for height and weight did not attenuate the relationship.

Individual SNPs explained between 0.2% and 0.5% of the variance in cortical vBMD. Regression models were constructed to determine the amount of variation explained by all significant and replicated SNPs. There was high linkage disequilibrium (LD) between the two SNPs in the HOXA gene region (rs6951180 and rs6964896; r2=0.691; D'=0.967) and the two SNPs in KLF10 (rs3133287 and rs1434278; r2=0.882; D'=1.000); thus, rs6964896 and rs1434278 were dropped from the regression modeling to minimize collinearity in the model. The 5 remaining SNPs in IFNAR2, NFATC1, SMAD1, HOXA and KLF10 explained 1.8% of the variance (adjusted r2) in cortical vBMD after accounting for age, clinic, population sub-structure, height and weight.

We also identified 255 SNPs in 75 genes that were associated with trabecular vBMD (Figure 4-2a). We confirmed an association with 12 of these SNPs in 9 genes and trabecular vBMD in the validation sample (Figure 4-2b). Specifically, associations with one SNP in ATF2 (rs4972738), BMP7 (rs6127983), FGF18 (rs313543), FLT1 (rs1408245), TGFB3 (rs7149264)

and THRB (rs1505289) and two SNPs in APC (rs459552 and rs4705573), BMP3 (rs2903746 and rs6814223) and RUNX1 (rs2834676 and rs2834694) were replicated in the validation sample (Figure 4-2b). In addition, SNPs in NTRK3 (rs6496469), SOX5 (rs2133345), TCF4 (rs618869) and TCF7 (rs30496) were significantly associated with trabecular vBMD in both the discovery and validation samples but the direction of association was inconsistent between samples.

Further analysis of the 12 consistently associated SNPs was conducted in the pooled sample. The most significant SNP association for trabecular vBMD was with rs2834694 in RUNX1 ($p=5.3x10-6$) which explained 1% of the variance in trabecular vBMD. Individuals with the less common AA genotype had a 14% higher trabecular vBMD than men with the CC genotype. Statistical adjustment for height and weight in addition to age, clinic and population sub-structure did not significantly attenuate the association between the 12 replicated SNPs and trabecular vBMD (Table 4-3).

Each of the 11 replicated SNPs individually explained between 0.3 and 1.0% of the variation in trabecular vBMD. None of the 12 replicated SNPs were highly correlated (even those in the same gene region) and all were entered into a regression model. After accounting for age, clinic, population sub-structure, height and weight, these 12 SNPs explained 4.0% of the variation in trabecular vBMD.

4.5 DISCUSSION

Studies in humans and animal models suggest that the genetic determinants of trabecular and cortical BMD may differ but few specific loci have been identified for these bone strength

related traits. The current study is the first, to our knowledge, to systematically investigate the association between common genetic variation in bone metabolism candidate genes and volumetric BMD in the trabecular and cortical bone compartments in humans. We identified several genetic variants that were associated robustly with cortical or trabecular vBMD at the femoral neck in a large cohort of older Caucasian men. Specifically, 7 SNPs located in 5 gene regions were consistently associated with cortical vBMD and 12 SNPs in 9 genes were consistently associated with trabecular vBMD in two independent samples of men from the same study cohort. No SNP consistently associated with one bone compartment was also associated with the other compartment in this analysis. These results suggest that loci for cortical and trabecular volumetric BMD at the femoral neck may be, at least to some extent, unique for each bone compartment.

To the best of our knowledge, associations with SNPs in IFNAR2, SMAD1 and KLF10 and cortical vBMD have not been described previously. IFNAR2 encodes a membrane receptor for type I interferons, interferon α and β(23). Type I interferon signaling is important in osteoclast regulation and bone resporption (24,25). The protein encoded by SMAD1 is a signal transduction molecule and transcriptional modulator of bone morphogenetic protein receptors(26). Targets genes of SMAD1 stimulate osteoblast differentiation and mineralized matrix formation(27). Krupple-like factor 10 (KLF10), also known as transforming growth factor beta (TGFB) inducible early growth response (TIEG), is expressed in osteoblasts and is induced by TGFB, bone morphogenetic proteins and epidermal growth factor(28). KLF10 expression in osteoblasts is critical for both osteoblast-mediated mineralization and osteoblast support of osteoclast differentiation (29,30). KLF10 knockout mice have decreased cortical bone thickness relative to wild-type controls and a significant decrease in osteocyte number (31).

A few of the SNPs associated with cortical vBMD were also associated with integral volumetric BMD at the femoral neck in a previous analysis of this cohort(16). For example, we observed an association between the rs6951180 variant in the Homeobox A (HOXA) gene cluster and femoral neck integral vBMD (16). Both SNPs associated with cortical vBMD in this study (rs6951180 and rs6964896) lie upstream of the HOXA gene cluster in the 5' flanking region of HOXA13. The HOXA genes are transcription factors involved in the patterning of the limbs during development and these genes may also be reactivated during fracture repair(32). Little is known about the possible role of HOXA genes in bone metabolism and maintenance of BMD in adults. Nevertheless, HOXA10, which lies approximately 45kb downstream of the SNPs associated in our study may contribute to osteoblastogenesis by regulating target genes for osteoblast differentiation and bone formation(33).

The less common G allele of rs177820 in NFATC1 was associated with lower cortical vBMD in this investigation. In our previous analysis, we identified a similar association of the G allele with lower lumbar spine vBMD but not femoral neck intergral vBMD (16). NFATC1 encodes nuclear factor of activated T-cells cytoplasmic 1, a transcriptional regulator of osteoclast differentiation and osteoclastogenesis(34). On the other hand, experiments in mice expressing a constitutively active NFATC1 suggest that it regulates bone mass by functioning in both osteoblasts and osteoclasts(35).

We also identified a novel association between SNPs in ATF2 and trabecular vBMD. ATF2 encodes activating transcription factor 2, a transcription factor that binds to cAMP response elements (CREs) and stimulates CRE-dependent transcription. The ATF family plays a role in the expression of skeletal-specific genes and skeletal tissue development(36). ATF-2 may

play a role in trabecular bone formation(37). Indeed, ATF-2 knockout mice lack normal-appearing trabeculae(38).

SNPs in genes encoding bone morphogenetic proteins BMP7 and BMP3, were also associated with trabecular vBMD in our analysis. Bone morphogenetic proteins (BMPs) direct skeletal patterning, chondrogenesis and bone formation(26). BMP3 is one of the most abundant BMPs in bone and BMP3 knockout mice have twice the trabecular bone as their wild-type littermates(39,40). BMP7 increases bone formation by promoting osteoblastic differentiation and clinical studies have demonstrated efficacy of recombinant human BMP7 in the treatment of tibial fractures(41-43).

A variant in the gene region encoding fibroblast growth factor 18 (FGF18) was also associated with trabecular but not cortical vBMD at the femoral neck. FGF18 may have a direct effect on osteoblast development in trabecular bone and coordinate both chondrogenesis in the growth plate and osteogenesis in trabecular bone(44). FGF18 null mice have delayed skeletal mineralization which is thought to be the result of delayed initiation of chondrocyte hypertrophy, decreased proliferation in the early stages of chondrogenesis, delayed skeletal vascularization and delayed osteoclast and osteoblast recruitment to the growth plate (45,46).

We also observed an association between an intronic SNP in the gene encoding thyroid hormone receptor beta (THRB) and trabecular vBMD. Thyroid hormone regulates bone turnover and mineralization in adults and is essential for skeletal development. Thyroid hormone action is mediated by thyroid hormone receptors, which act as hormone inducible transcription factors to regulate expression of thyroid hormone-responsive target genes. Although thyroid hormone receptor alpha appears to be the predominant hormone receptor in bone, mRNA and protein for thyroid hormone receptor beta are present in growth plate chondrocytes and

osteoblasts at sites of endochondral and intramembranous ossification(47-50). Furthermore, juvenile thyroid hormone receptor beta knockout mice have advanced endochondral and intramembranous ossification and increased bone mineral deposition, whereas adult animals have osteoporosis with reduced trabecular and cortical bone, reduced mineralization and increased osteoclast numbers and activity(51). Thyroid hormone receptor beta is also critical for the determination of both the set-point of the hypothalamic pituitary thyroid axis and the levels of circulating thyroid hormones. Thus, we are unable to conclude if the association of THRB polymorphisms and trabecular BMD in the current study might be mediated through direct skeletal actions of THRB or are indirectly mediated through circulating thyroid hormone levels.

Two intronic SNPs in the gene encoding the runt homology domain transcription factor, RUNX1, were associated with trabecular vBMD. The Runx family of transcription factors plays a fundamental role in organ development and cell differentiation. During skeletal development, Runx1 is thought to play a role in osteochondroprogenitor cell differentiation and in mediating early events of endochondral and intramembranous bone formation(52-54). Polymorphisms in another Runx family member, RUNX2, have been associated with BMD and bone size but we are unaware of any previous reports of an association between RUNX1 SNPs and BMD (55-59). We were unable to confirm an association between RUNX2 SNPs and trabecular or cortical vBMD at the femoral neck in this study.

The two variants in the gene encoding adenomatous polyposis coli (APC) (rs459552 and rs4705573) that were associated with trabecular vBMD in the present analysis were also associated with integral vBMD at the femoral neck in our prior analysis (16). Adenomatous polyposis coli (APC) is involved in WNT signaling, is expressed in osteoblasts and osteoclasts, and mice with osteoblast specific APC deletion have increased bone formation(60-64). The

association with rs459552 is of particular interest since this is a non-synonymous variant that results in a change from an asparagine residue to a valine residue and has been identified in studies of familial adenomatous polyposis(65). This SNP is located in the B-catenin down regulation domain of APC and could potentially regulate WNT signaling (65,66). Similarly, a SNP in FLT1 (rs1408245) was associated with femoral neck trabecular vBMD in the current analysis and with femoral neck integral vBMD in our previous analysis (16). FLT1 encodes the cell-surface receptor for vascular endothelial growth factor that is involved in osteoclastogenesis and osteoblast differentiation(67-71). FLT1 null mice have lower trabecular bone volume which is consistent with our findings (72). We also identified a SNP upstream of transforming growth factor beta 3 (TGFB3) that was associated with trabecular vBMD. TGFB3 is involved in bone formation and increased levels of circulating TGFB3 have been associated with osteoporosis in women(73-75). Although no association has been previously described between SNPs in TGFB3 and BMD, an intronic variant (rs2268624) was associated with ossification of the posterior ligament of the spine(76).

The current findings are consistent with an emerging model whereby genetic loci for BMD may be at least to some extent specific for cortical and trabecular bone. For example, genome-wide searches in mice have identified quantitative trait loci that may be distinctly associated with either cortical or trabecular vBMD (5,8). Furthermore, family studies have found a low genetic correlation between trabecular and cortical vBMD suggesting different genetic contributions for each bone compartment (11). Similarly, in the current investigation none of the replicated genes or SNPs that had consistent statistical associations were associated with both cortical and trabecular vBMD at the femoral neck.

We were able to identify more genetic associations and explain a greater fraction of phenotypic variation for trabecular than for cortical vBMD. It is possible that the genetic contribution to trabecular vBMD is greater than for cortical vBMD. Indeed, heritability studies in humans report a lower heritability for cortical vBMD (17-42%) than for trabecular vBMD (59-73%) but the average age of participants was younger than in our study (9-11). However, the cortical rim of the femoral neck is thin and this may have increased measurement error and consequently decreased statistical power at this skeletal region. Future studies of the appendicular skeleton in regions with a larger cortical area (for example, at the proximal tibia) may help to minimize this issue. Our candidate gene selection was also based on several informatics resources, but nevertheless may have been biased towards genes influencing trabecular bone. Future studies of genetic factors for trabecular and cortical vBMD that utilize hypothesis-free methods (such as genome-wide investigations) may yield further insight.

SNPs in several additional genes were significantly associated with either trabecular or cortical vBMD in both the validation and discovery samples but the direction of the association was inconsistent (IGF1R, NTRK, SOX5, TCF4, TCF7). To our knowledge, only IGF1R has been investigated in prior candidate gene association studies of osteoporosis related phenotypes(77). Although the evidence for these associations is not as strong, there are characterized biological scenarios for this phenomenon of different directions of association and these genes may warrant further examination(78,79).

We were unable to document an association between SNPs in several widely studied candidate genes (e.g., COL1A1, ESR1, LRP5, VDR) and trabecular or cortical volumetric BMD in the current study(80-83). Further, two recent genome-wide association studies identified significant associations with variation near the LRP5, RANKL, TNFRSF11B,

ESR1, and WNT4 genes and BMD measured by DXA(84,85). There are several reasons why we may not have been able to replicate these previous associations. First, our study included only men, whereas these past studies have focused primarily on women. In addition, most of these prior candidate gene studies measured integral areal BMD by DXA as opposed to cortical and trabecular volumetric BMD by QCT. Finally, our study was able to detect a SNP association that explained at least 0.8% of the variation in BMD in the discovery sample with 80% statistical power at alpha = 0.05. Thus, we cannot exclude the possibility of a weaker association between SNPs in or near the ESR1, COL1A1, VDR, TNFRSF11B, RANKL, WNT4 or LRP5 genes and volumetric BMD at the femoral neck in older men.

Although the heritability of volumetric BMD is high (estimates range from 40-85%), the amount of phenotypic variation in vBMD explained by SNPs in the current study was small (1.7% for cortical vBMD and 4.0% for trabecular vBMD). Although small, the fraction of variation explained by these SNPs is consistent with the effect size reported in recent genome-wide association studies of skeletal related traits such as BMD and height (84-86). For example, in a recent genome-wide association study of DXA measures of areal BMD, 0.6% and 0.2% of the phenotypic variation in lumbar spine and femoral neck BMD was explained by two SNPs in two genes(84). Future studies may need to consider other types of polymorphisms such as insertion-deletion mutations, copy number variants, and rare SNPs (<5% MAF) as well as interactions between genes and environmental factors in order to better account for the phenotypic variation in vBMD.

Our analysis focused on older Caucasian men and our findings may not be generalizable to other populations. Additional genotyping in younger men, ethnically diverse populations and in women would be useful to further confirm and extend these SNP association findings.

Tagging SNP selection was based on Phase I of the International Haplotype Map Project (HapMap) and more recent releases of the HapMap project (Phase II) would have provided a larger SNP reference panel to select tagging SNPs from. However, the SNPs selected for the current analysis captured 64% of the common SNPs (≥5% MAF) in Phase II of HapMap with a mean max r2 of 0.97. We also cannot assess the impact that rare variants might have on trabecular and cortical vBMD in our study since we restricted our analysis to tagging SNPs with MAF ≥5% and potentially functional SNPs with a MAF of ≥1%. Finally, it is unclear if the SNPs identified in the current analysis have functional consequences or are merely in linkage disequilibrium with the causal variant(s). Additional studies will be needed to confirm and refine the current association signals and to identify the causal variants involved.

In conclusion, this is the first large-scale investigation of the potential genetic determinants of cortical and trabecular volumetric BMD in humans at a clinically relevant skeletal site, assessed and adjusted for potential population stratification, and identified several novel and reproducible genetic associations using a two-stage internal replication design. Although additional studies are needed to confirm and extend our findings, the current analysis suggests that the genetic factors contributing to cortical and trabecular volumetric BMD among older men may be at least to some extent unique for each bone compartment.

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4.7 TABLES AND FIGURES

Table 4-1 Characteristics of genotyping samples.

	Discovery Sample N=822	Validation Sample N=1155	Pooled Sample N=1977
Age (years)	73 (5.7)	74 (6.0)	74 (5.9)
Height (cm)	173.6 (6.8)	174.9 (6.7)*	174.3 (6.7)
Weight (kg)	85.3 (14.1)	82.9 (12.5)*	83.9 (13.2)
BMI (kg/m^2)	28.3 (4.1)	27.1 (3.6)*	27.6 (3.8)
Femoral Neck Cortical Volumetric BMD (g/cm3)	0.532 (0.055)	0.520 (0.065)*	0.525 (0.061)
Femoral Neck Trabecular Volumetric BMD (g/cm^3)	0.086 (0.044)	0.062 (0.040)*	0.072 (0.043)

***Significant difference between discovery and validation sample ($p<0.001$)**

Table 4-2 Significant SNP associations with cortical volumetric BMD at the femoral neck.

				Discovery Sample		Validation Sample		Pooled Sample				
				adjustment 1[b]		adjustment 1[b]		adjustment 1[b]		adjustment 2[c]		
Gene	SNP	Allele	Frequency[a]	β	P-value	β	P-value	β	P-value	β	P-value	r^{2} [d]
HOXA[e]	rs6951180	A→G	0.13	0.013	0.001 add	0.027	0.034 rec	0.025	0.017 rec	0.023	0.026 rec	0.002
HOXA[e]	rs6964896	C→A	0.13	0.012	0.003 add	0.027	0.048 rec	0.024	0.025 rec	0.023	0.036 rec	0.002
IFNAR2	rs2834160	A→G	0.17	0.011	0.002 add	0.022	0.025 rec	0.023	0.001 rec	0.024	0.001 rec	0.005
NFATC1	rs177820	A→G	0.36	-0.009	0.001 add	-0.010	0.034 rec	-0.013	$4x10^{-4}$ rec	-0.012	0.001 rec	0.005
SMAD1	rs1874572	A→C	0.33	0.017	0.003 rec	0.005	0.046 add	0.011	0.005 rec	0.011	0.006 rec	0.004
KLF10	rs1434278	T→A	0.23	0.022	0.013 rec	0.006	0.0366 add	0.006	0.002 add	0.006	0.001 add	0.005
KLF10	rs3133287	G→C	0.17	0.038	0.001 rec	0.006	0.055 add	0.007	0.001 add	0.007	0.001 add	0.005

Additive and recessive models were tested for each SNP and the regression parameter and p-value from the most significant genetic model (additive or recessive) is shown.
add: p-value from the additive model; rec: p-value from recessive model
a)Minor allele frequency in the pooled sample
b) Adjustment 1: Age, clinic site, population sub-structure
c) Adjustment 2: Age, clinic site, population sub-structure, height, body weight
d) Amount of variation explained after adjusting for age, site, population sub-structure, height, body weight
e) HOXA genes were tagged as a cluster. They include HOXA1, HOXA2, HOXA3, HOXA4, HOXA5, HOXA6, HOXA7, HOXA9, HOXA10, HOXA11, HOXA13

Table 4-3 Significant SNP associations with trabecular volumetric BMD at the femoral neck

				Discovery Sample		Validation Sample		Pooled Sample				
				adjustment 1[b]		adjustment 1[b]		adjustment 1[b]		adjustment 2[c]		
Gene	**SNP**	**Allele**	**Frequency[a]**	***β***	***P-value***	***β***	***P-value***	***β***	***P-value***	***β***	***P-value***	$r^{2\,d}$
APC	rs459552	T→A	0.23	0.006	0.018 add	0.004	0.049 add	0.004	0.005 add	0.004	0.006 add	0.004
APC	rs4705573	A→G	0.47	-0.007	0.048 rec	-0.007	0.012 rec	-0.007	0.002 rec	-0.007	0.002 rec	0.005
ATF2	rs4972738	G→A	0.37	-0.011	0.012 rec	-0.007	0.054 rec	-0.009	0.001 rec	-0.009	0.001 rec	0.005
BMP3	rs2903746	A→T	0.18	0.006	0.022 add	0.005	0.012 add	0.005	0.003 add	0.005	0.003 add	0.004
BMP3	rs6814223	G→A	0.13	0.007	0.038 add	0.006	0.017 add	0.005	0.007 add	0.005	0.007 add	0.004
BMP7	rs6127983	A→G	0.36	-0.009	0.053 rec	-0.007	0.040 rec	-0.007	0.008 rec	-0.007	0.007 rec	0.004
FGF18	rs9313543	G→A	0.19	-0.017	0.029 rec	-0.005	0.018 add	-0.004	0.013 add	-0.004	0.019 add	0.003
FLT1	rs1408245	C→G	0.17	0.010	0.008 rec	-0.006	0.003 add	-0.016	0.002 rec	-0.016	0.003 rec	0.004
TGFB3	rs7149264	A→C	0.15	0.006	0.022 add	0.004	0.050 add	0.005	0.008 add	0.005	0.011 add	0.003
THRB	rs1505289	A→G	0.43	-0.004	0.048 add	-0.004	0.034 add	-0.004	0.003 add	-0.004	0.003 add	0.004
RUNX1	rs2834676	G→A	0.42	0.005	0.030 add	0.010	0.002 rec	0.004	0.003 add	0.007	0.002 rec	0.005
RUNX1	rs2834694	C→A	0.49	0.006	0.007 add	0.011	1.4×10^{-4} rec	0.010	5.3×10^{-6} rec	0.010	4.2×10^{-6} rec	0.010

Additive and recessive models were tested for each SNP and the regression parameter and p-value from the most significant genetic model (additive or recessive) is shown.
add: p-value from the additive model; rec: p-value from recessive model
a)Minor allele frequency in the pooled sample
b) Adjustment 1: Age, clinic site, population sub-structure
c) Adjustment 2: Age, clinic site, population sub-structure, height, body weight
d) Amount of variation explained after adjusting for age, site, population sub-structure, height, body weight

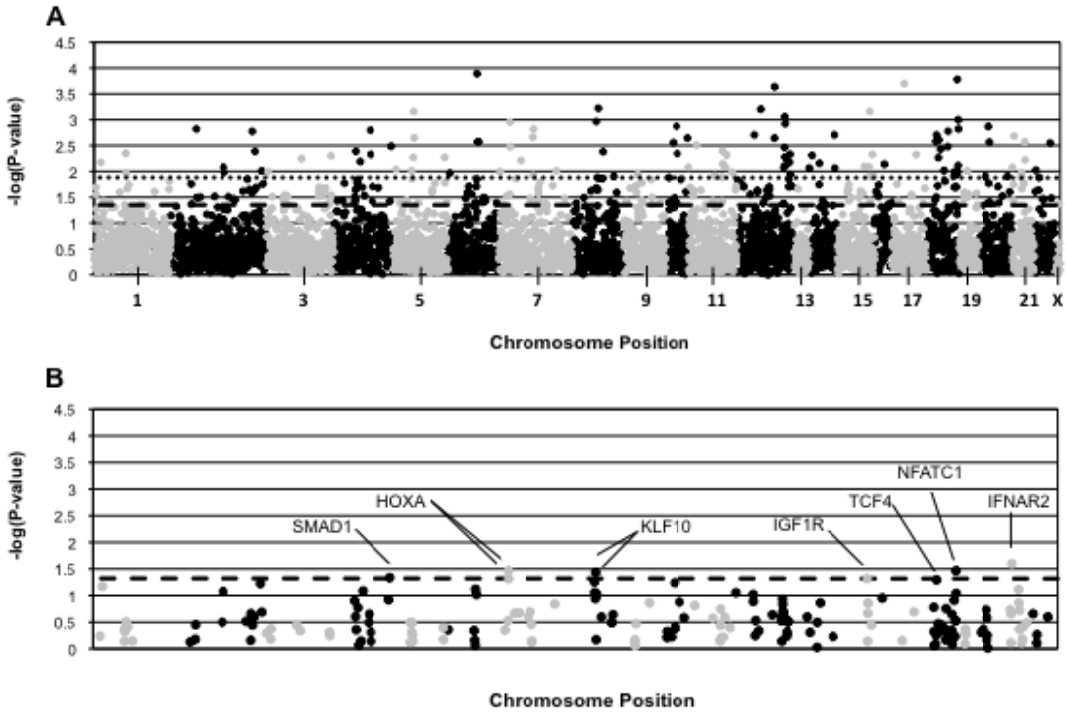

Figure 4-1 SNP association results for femoral neck cortical volumetric BMD.

Association results for cortical volumetric bone mineral density are presented for the first phase of genotyping (discovery sample) in *Figure 4-1a* and in the validation sample (*Figure4- 1b*). Specifically, the -log of the p-value observed is presented on the y-axis. The most significant result of the two models tested (either additive or recessive) is presented for each SNP. The SNPs are ordered across the x-axis by chromosome and the base pair position on the chromosome. Odd numbered chromosomes and the X chromosome are presented in light grey. Even numbered chromosomes are presented in dark grey. In *Figure4- 1a* the dark dashed line represents p=0.015 and the dotted line represents p=0.05. The dotted line in *Figure4- 1b* represents p=0.05 and SNPs with p-values ≤0.05 are labeled with the gene symbol that they lie in.

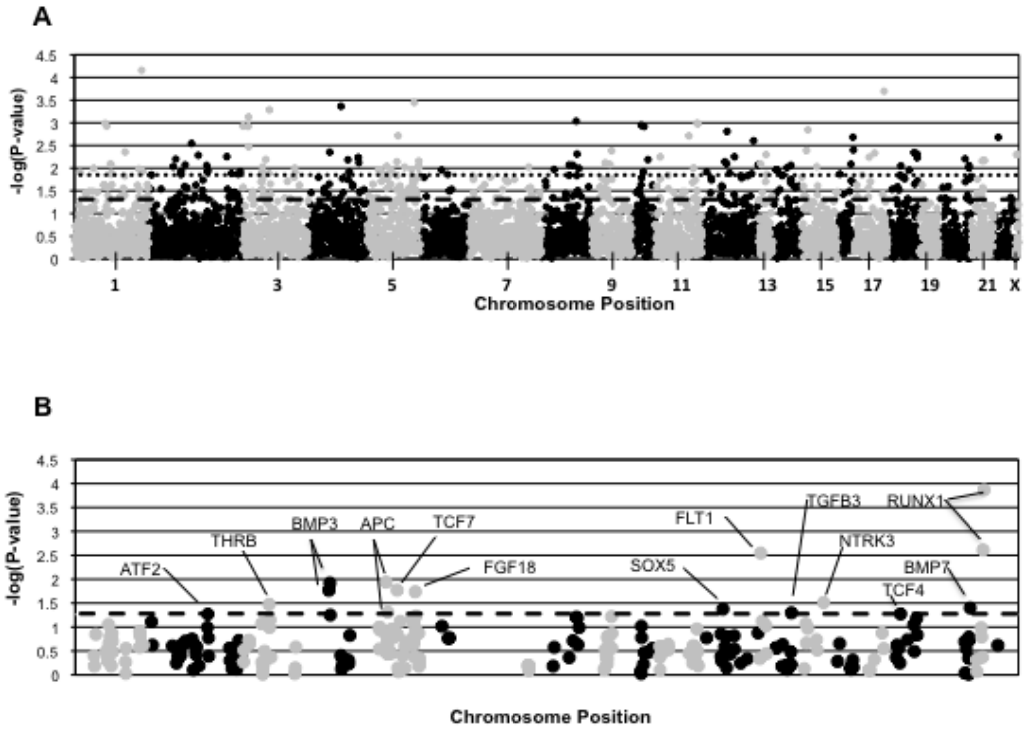

Figure 4-2 SNP association results for femoral neck trabecular volumetric BMD

Association results for the lumbar spine are presented for the discovery (*Figure 4-2a*) and validation sample (*Figure 4-2b*). Specifically, the –log of the p-value observed is presented on the y-axis and SNPs are ordered across the x-axis by chromosome and base pair position. The most significant result of the two models tested (either additive or recessive) is presented for each SNP. Odd numbered chromosomes and the X chromosome are presented in light grey. Even numbered chromosomes are presented in dark grey. In *Figure4- 2a* the dark dashed line represents p=0.015 and the dotted line represents p=0.05. The dotted line in *Figure 4-2b* represents p=0.05 and SNPs with p-values ≤0.05 are labeled with the gene symbol that they lie in.

5.0 ARTICLE 3: EVALUATION OF GENE-GENE AND GENE-ENVIRONMENT INTERACTIONS IN THE WNT PATHWAY WITH BONE MINERAL DENSITY

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5.1 ABSTRACT

The WNT/ß-catenin Signaling Pathway is important in skeletal development and bone regeneration throughout the lifespan. A number of associations between genetic variation in WNT pathway members and bone mineral density (BMD) have been identified, but little is known about how interactions between WNT pathway genes or between WNT pathway genes and the environment influence BMD. In this study, 148 SNPs in 28 WNT pathway members were interrogated for significant gene-gene and gene-environment interactions with volumetric BMD at the lumbar spine and femoral neck in a sample of older Caucasian men from the Osteoporotic Fractures in Men Study (MrOS). Specifically, interactions between SNPs and physical activity and interactions between SNPs and body weight were examined because of the hypothesized role of WNT signaling in the skeletal response to mechanical loading. An interaction between a SNP in SFRP4 (rs2722303) and body weight was associated with lumbar spine volumetric BMD. A previously reported interaction between physical activity level and SNPs in LRP5 with BMD was also confirmed. In addition, a number of interactions between genotypes in WNT pathway genes were identified for both the lumbar spine and femoral neck. Although additional work is needed, these findings underscore the importance of evaluating genetic variation in the context of other genes and the environment when evaluating the genetic basis for BMD.

5.2 INTRODUCTION

The WNT/ß-catenin signaling pathway is an important signal transduction pathway involved in skeletal development and post-natal bone accrual. This pathway is thought to influence bone mineral density in a number of ways including the regulation of osteoblast differentiation and proliferation and the control of apoptosis in osteoblasts and osteoclasts(1-4).

Evidence is also building that WNT signaling is involved in bone's response to mechanical loading. Mechanical loading increases osteoblast proliferation and activity to promote bone formation and to adapt to the strain put on the bone(5,6). In vivo mouse models demonstrate prominent WNT signaling in the osteocytes, the cells thought to be the mechano-sensors of bone(7). Knockout mouse models for low density lipoprotein receptor-related protein 5 (LRP5), an important co-receptor in WNT signaling, have a suppressed response to mechanical loading compared to control mice(8). A study in humans observed statistically significant interactions between LRP5 variation and physical activity level on BMD (9).

Linkage and genetic association studies first identified associations with variants in the LRP5 gene and familial low and high BMD conditions and later with normal variation in BMD (10-13). Associations between genetic variation in a number of WNT pathway genes (including DKK2, FZD1, LRP1, LRP6, SFRP1, SOST, WNT3a and WNT10b) and bone mineral density have been subsequently reported(14-17). Although population genetic studies of WNT pathway members are becoming more common, little is known about the role of epistatic (or gene-gene) interactions between WNT pathway members and BMD.

In order to better understand how SNPs in WNT pathway members may interact with each other and with environmental factors, we systematically screened SNPs in 28 WNT pathway genes for interaction effects with each other and with indices of mechanical loading

(i.e., physical activity, body weight) on volumetric bone mineral density (vBMD) in a population of older Caucasian men. Since prior evidence of an interaction between physical activity level and LRP5 SNPs exists, an a priori analysis of this gene was completed.

5.3 METHODS

5.3.1 Study Population

Participants were Caucasian men selected from the Osteoporotic Fractures in Men Study (MrOS). Briefly, MrOS is a prospective cohort study of 5,995 community-dwelling men age ≥ 65 from 6 clinical centers across the United States (18,19). To be eligible for recruitment into MrOS, men had to be ambulatory and not have had bilateral hip replacement.

Caucasian MrOS participants with volumetric bone mineral density (vBMD) that were not taking bone-altering medications (androgens, anti-androgens, oral corticosteroids or osteoporosis treatment) were selected as part of a two-phase, large-scale candidate gene study(20). The first phase of genotyping was carried out on a discovery sample, which included 862 Caucasian men with hip or spine vBMD measures that were selected without regard to their BMD level from the Minneapolis, MN and Pittsburgh, PA clinic sites. SNPs showing promising findings for volumetric BMD traits of interest were tested for replication in a validation sample consisting of 1156 additional men from the four other MrOS clinic sites (Birmingham, AL; Palo Alto, CA; Portland, OR and San Diego, CA).

5.3.2 Participant Characteristics

At the baseline visit, participant characteristics including age, medical history and medication usage were recorded. Height was measured by Harpenden stadiometer and weight was measured by balance beam scale with participants wearing light clothing and no shoes.

Physical activity was measured using the Physical Activity Scale for the Elderly (PASE)(21). This is a validated scale that is designed to assess physical activity in epidemiological studies of participants 65 years of age and older. PASE consists of 12 questions that assess the leisure, household and occupational activities over the previous week. Each of the questions is weighted using participant report of the amount of time spent for each activity and previously derived weights from physical activity logs and motion sensor data (22).

5.3.3 Gene and SNP Selection

SNP variation in WNT pathway genes was identified from a previous candidate gene study(20). The previous study investigated 4608 SNPs in 373 genes in a discovery sample of 862 men. The 4608 SNPs in the previous study were either tag SNPs selected to cover the common variation across the candidate gene region (including the transcript, 30kb upstream and 10kb downstream) or were potentially functional SNPs. Tagging SNPs were selected using a pair-wise correlation method called Hclust ($r2 \geq 0.80$)(23). Potentially functional SNPs that were non-synonymous and had a minor allele frequency (MAF) >1%, putative exon splice enhancers and promoter variants (MAF >2%) were identified using either the PupaSNP (pupasuite.bioinfo.cipf.es/) or Promolign (polly.wustl.edu/promolign/main.html) databases (24,25). Replication of the most promising SNPs ($p < 0.015$ or $p < 0.05$ and in the same gene region as a SNP with $p < 0.015$) was

completed in a second validation sample. This validation sample consisted of 1156 Caucasian men from the Birmingham, AL; Palo Alto, CA; Portland, OR; and San Diego, CA

The WNT pathway had been targeted for genotyping in this previous study. 148 SNPs in 28 genes from the WNT pathway were genotyped in both the discovery and validation sample and were included in this analysis (Figure 1). However, since only SNPs that had promising associations were included in the validation study, the WNT pathway members are not comprehensively tagged in this analysis. Compared to Caucasian reference data from Phase 2 of the International HapMap Project, the SNPs in our analysis were able to cover an average of 33.0% (range 4.4%-90.7%) of the common variation (MAF >5%) in the reference database (Table 5-1) (26).

For LRP5, only four SNPs were genotyped in both the discovery and validation genotyping samples (rs312018, rs314756, rs634918, and rs676318). Since an interaction effect of LRP5 genotype and physical activity on BMD was previously described, additional analysis of the 12 LRP5 SNP variants from the more comprehensive, discovery set of SNPs was conducted.

5.3.4 Volumetric Bone Mineral Density

Quantitative computed tomography (QCT) was used to measure vBMD of the central skeleton in a subset of men in the MrOS cohort. Specifically, the first 65% of the cohort and all non-white men were referred for QCT scans. QCT scans of the lumbar spine and pelvis were used to obtain vBMD for two anatomical regions of interest (ROIs); the second lumbar vertebra and the femoral neck.

The lumbar spine scan was obtained from 5 mm above the L1 superior endplate to 5 mm below the L2 inferior endplate and the ROI included the entire second lumbar verebra excluding the transverse process. The images were acquired using a setting of 120kBP, 150 mA, 1 mm slice thickness and 512x512 matrices. The hip scan was obtained from the femoral head to 3.5 cm below the lesser trochanter at settings of 80 kVp, 280 mA, 3-mm slice thickness, and 512 x 512 matrix in spiral reconstruction mode. The femoral neck ROI is from the minimum cross-sectional area to the point 25% towards the maximum cross-sectional area where cross-sectional area was measured along the neutral axis. The images were acquired on a GE Prospeed at the Birmingham clinic, a GE Hispeed Advantage at the Minneapolis clinic, a Philips MX-8000 at the Palo Alto clinic, a Siemans Somatom +4 at the Pittsburgh clinic, either a Philips CT-Twin or Toshiba Acquilion at the Portland site, and a Picker PQ-5000 in San Diego. Differences between the clinic sites exist and are statistically adjusted for in all analyses. QCT images were processed at the University of California at San Francisco using a standardized protocol. Each participant scan included a calibration standard of three hydroxyapatite concentrations (150, 75 and 0 mg/cm3) and these were used to convert between Hounsfield units and vBMD.

5.3.5 Statistical Analysis

Participant characteristics including age, physical activity, weight, height, BMI and vBMD were compared between the discovery and validation samples using analysis of variance. Previous analyses of this data set have revealed little population stratification, yet we accounted for any fine scale population sub-structure by employing a principal components method calculated using uncorrelated SNPs ($r < 0.2$) from the larger candidate gene study (27,28).

The analyses of genotype interactions with physical activity were carried out using linear regression modeling that included terms for age, clinic, population sub-structure, SNP genotype (coded as the number of minor alleles), PASE score (continuous variable) and the multiplicative term between SNP genotype and PASE score. The analyses for genotype interactions with body weight were also carried out using linear regression modeling and these models included the terms for age, clinic, population sub-structure, height, SNP genotype (coded as the number of minor alleles), body weight and the multiplicative term between SNP genotype and body weight. For the systematic analysis of genotype-environment interactions, analyses were done in the discovery sample and statistically significant interactions ($p<0.05$) were replicated in the validation sample. SNPs that were significant in both the discovery and validation sample and that had a consistent direction of association (i.e., having either a negative or positive regression coefficient for both the discovery and validation sample) were considered to be replicated. Replicated findings were further examined in the pooled sample of 2018 participants from the discovery and validation samples. The replicated findings are presented graphically by genotype and tertile of body weight.

Although genotype-environment interaction analyses were modeled in the same way, the full set of 12 LRP5 SNPs was only available in the discovery sample, so significant associations could not be replicated in the validation sample. Significant interactions between PASE and LRP5 genotype were further graphed by genotype and tertile of physical activity. Tertiles of PASE in the discovery sample were used for this analysis.

Analysis of genotype-by-genotype interactions among WNT pathway genes was completed using regression modeling that included terms for both SNP genotypes (coded as the number of minor alleles), the multiplicative interaction term between the two SNP genotypes,

age, clinic and population sub-structure. Analyses for genotype by genotype interactions were initially completed in the discovery sample and statistically significant interactions ($p<0.05$) were replicated in the validation sample. Interactions that were statistically significant in both the discovery and validation samples ($P<0.05$) and that had a consistent direction of association were considered replicated.

5.4 RESULTS

There was no difference between the discovery and validation sample for PASE score ($p=0.73$), but men in the validation sample weighed less than men in the discovery sample ($p <0.001$) (Table 5-2). Age was not different between the discovery and validation samples, but men in the validation were slightly taller, had lower vBMD at the femoral neck and lumbar spine ($p<0.001$).

5.4.1 Interactions between WNT Pathway Members and Physical Activity and Body Weight

Interactions between PASE physical activity score and the 148 SNPs in WNT pathway genes were tested for their association with integral vBMD at the femoral neck and lumbar spine. In the discovery sample, 7 SNPs in 6 genes had significant interaction effects for femoral neck vBMD, but only one SNP (rs10488900 in DKK2) that had a significant interaction in the discovery sample ($p=0.040$) was also significant in the validation sample ($p=0.027$). However, the direction of the association was not consistent in the two samples. For the lumbar spine, 5 SNPs in 4 genes had significant findings in the discovery sample, but only rs1420731 was also

significant in both the discovery (p=0.048) and the validation sample (p=0.047) but the direction for this association was also not consistent.

Interaction effects between body weight and the 148 SNPs in the WNT pathway genes were also examined for their association with integral vBMD at the femoral neck and lumbar spine. 10 SNPs in 6 genes had statistically significant interaction effects with weight for femoral neck vBMD in the discovery sample, but none of these associations were statistically significant in the validation sample. For the lumbar spine, 9 SNPs in 7 genes had statistically significant interactions with body weight in the discovery sample. One SNP in WNT5b (rs3926367) had a statistically significant interaction with body weight in the discovery (p=0.015) and validation samples (p=0.019) but did not have a consistent direction of association. A SNP downstream of SFRP4 (rs2722303) showed a consistent statistically significant interaction effect with body weight on lumbar spine vBMD in the discovery (p=0.050), validation (p=0.031) and pooled samples (p=0.009). Lumbar spine vBMD means from the pooled analysis adjusted for age, clinic, population sub-structure and height are presented by tertile of body weight and genotype for this SNP (Figure 2). Higher body weight was associated with higher lumbar spine vBMD for the GG and GC genotypes, but weight was not associated with vBMD among men with the CC genotype (pairwise comparisons between tertiles within this genotype group are all >0.40). Furthermore, the C allele was associated with higher vBMD for those men in the lowest tertile of body weight (p= 0.005 for GG vs. CC genotype), but was not associated with vBMD in the second tertile of body weight (p=0.44 for GG vs. CC genotype) and the C allele was associated with lower vBMD for those men in the highest tertile of body weight (p=0.04 for GG vs. CC genotype).

5.4.2 Interaction Effects of LRP5, Physical Activity and Body Weight

Only four SNPs in the LRP5 gene were genotyped in both the discovery and validation sample. However, an interaction between SNPs in this gene and physical activity had previously been described; thus, we expanded our analysis to investigate all 12 of the LRP5 SNPs genotyped in the discovery sample (Figure 5-3) (9). These 12 SNPs covered a much higher proportion of the genetic variation (52% coverage compared to Phase II of the HapMap Project) than the 4 SNPs genotyped in both the discovery and validation samples (14% coverage)(9). Of the 12 LRP5 SNPs, no SNP had a significant main effect association with vBMD using linear regression analysis to adjust for age and clinic.

There were nominally statistically significant interactions between PASE score and LRP5 SNPs (Figure 5-4A). Specifically, there was a statistically significant interaction between PASE score and two SNPs in LRP5 (rs638051 and rs643892) with volumetric BMD at both the femoral neck and lumbar spine. Men with the common genotype (AA) of rs643892 had lower femoral neck (p=0.010) and lumbar spine (p=0.033) vBMD with increased physical activity, but men with the less common genotype (TT) had higher vBMD with increased physical activity (Figure 5-5). A similar pattern was observed for rs638051 (Figure 5-6) with men possessing the common genotype (AA) having lower vBMD with increased physical activity and men with the less common genotype (GG) having higher vBMD with increased physical activity.

Interactions between LRP5 SNPs and body weight were also examined (Figure 5-4b). Significant interactions between rs634918 and body weight and between rs312018 and body weight were observed for femoral neck vBMD (p=0.009 and p=0.045 respectively). For lumbar spine vBMD, statistically significant interactions were observed between rs587397 and body weight (p=0.031) and between rs643892 and body weight (p=0.037). Only rs643892 had

statistically significant interactions with PASE score and body weight, but this statistically significant interaction was only observed for lumbar spine vBMD.

5.4.3 Gene-Gene Interactions Between WNT Pathway Members

In total, 9,887 pair-wise interactions between SNPs in 28 different WNT pathway genes were tested for integral vBMD in the femoral neck and lumbar spine. Interactions were tested in both the discovery and validation samples. In total, 23 pairs of SNPs in 19 pairs of genes had statistically significant interactions ($p<0.05$) in both the discovery and validation sample for femoral neck vBMD when adjusted for age, clinic and population sub-structure. Specifically, 22 pairs of SNPs in 16 pairs of genes also had statistically significant interactions after adjusting for age, clinic and population sub-structure for lumbar spine vBMD ($P<0.05$). These findings are summarized in Figure 5-7.

There were a number of interactions that were statistically significant in both the discovery and validation samples, but that did not have a consistent direction of association. For the lumbar spine and femoral neck combined, there were 17 of these SNP pairs that showed statistically significant but inconsistent associations.

Consistent, replicated interactions were observed for both femoral neck and lumbar spine vBMD. For the femoral neck, 15 SNP pairs within 11 pairs of genes were associated with vBMD when adjusted for age, clinic and population-substructure. Nine of the SNP pairs (across 7 pairs of genes) were statistically significant after additional adjustment for height and body weight. One of the SNP pairs in the LRP5-DVL2 interaction (rs634918 and rs222851), the two SNP pairs in the WNT5A-LRP6 interaction (rs11918967 and rs4763794; rs11918967 and rs4477532), the WNT5A-KREMEN1 interaction (rs1191897 and rs134665), the SFRP2-SOST

interaction (rs12645134 and rs1877632) and the FZD1-WNT11 interaction (rs6465306 and rs7933711) were no longer statistically significant after additional adjustment for height and body weight. The statistically significant interactions for the femoral neck are presented in Table 5-3.

Statistically significant interactions were also detected for lumbar spine vBMD (Table 5-4). In the model adjusted for age, clinic and population sub-structure, 13 pairs of SNPs within 8 pairs of genes were statistically significantly associated with vBMD. All 13 pairs of SNPs remained statistically significant with additional adjustment for height and body weight.

5.5 DISCUSSION

We identified several statistically significant and novel interactions between WNT pathway genes and indices of mechanical loading as well as evidence of gene-gene interactions within the WNT pathway. In particular, we confirmed a previous observation that SNP variation in the LRP5 gene modifies the relationship between physical activity and bone mineral density(9). Although WNT pathway genes have received considerable attention for their importance in BMD variation among individuals, the current analyses suggest that genotypes in WNT pathway genes may have context specific effects on BMD.

A statistically significant interaction between body weight and a SNP in the gene encoding secreted frizzled-related protein 4 (SFRP4) was associated with lumbar spine vBMD. SFRP4 is one member of a family of WNT pathway antagonists that bind WNT molecules to inhibit WNT signaling(29). Experiments using a low bone mass mouse model indicate that recombinant SFRP4 inhibits osteoblast formation, and mice over-expressing SFRP4 in

osteoblasts have suppressed bone formation, decreased bone mass and differences in trabecular micro-architecture(30,31). Mechanical loading experiments in mice showed that expression of SFRP4 was not increased after mechanical loading in wild-type mice but was increased after loading in mice carrying a high bone mass mutation in the LRP5 gene(32). Expression of other WNT pathway members (including WNT10b) was increased after mechanical loading in both wild-type and LRP5 mutant mice(32). Although mechanical loading did not directly change the expression of SFRP4, it may change the expression pattern of WNT molecules. The statistically significant interaction between body weight and rs2722303 in SFRP4 could be part of a higher order interaction.

Substantial evidence of genetic associations with SNPs in LRP5 and BMD exists(12,13,33), but there is conflicting information about the association of SNPs with BMD in older Caucasian men (9,20,34). We did not detect an association with LRP5 SNPs and vBMD in older Caucasian men(20) which is consistent with the analysis of areal BMD completed by Kiel et al. (9). However, another large study of older Caucasian men detected a statistically significant association between LRP5 SNPS and BMD that was stronger in men than in women(34). Although all three of these populations are Caucasian, the van Meurs study is a European cohort while the other two are from the United States. Differences in environmental exposures (such as average physical activity level over the lifespan) could be responsible for the differences between populations. We detected a statistically significant interaction between physical activity and LRP5 SNPs as did Kiel et al. We detected this interaction with two SNPs in intron 5 of the LRP5 gene, but the previous investigation observed an interaction between coding SNPs in exon 10 and exon 18. Additional evidence for the relationship between mechanical loading and LRP5 exists in animal studies. LRP5 null mice fail to increase bone mass in response to mechanical

loading although they are able to recruit osteoblasts normally(8). The interaction between physical activity and LRP5 genetic variation with BMD has been confirmed in two populations of older men and is supported by animal studies, but additional work is needed to understand the mechanisms underlying these interactions. A comprehensive analysis including more LRP5 variants and ethnically diverse populations of different ages will help clarify the relationship between physical activity and LRP5 in humans.

We observed a number of gene-gene interactions for vBMD, but did not adjust for multiple comparisons. With a type I error rate of 0.05 and 9,887 statistical tests in the discovery sample we would expect to observe 494 significant associations by chance. Following up on those 494 findings with a type I error rate of 0.05, 25 significant associations would be expected by chance, which is comparable to the number of interactions we detected. One of the most robust interactions was between an intronic SNP (rs6827902) in DKK2 and a SNP upstream of the SOST gene (rs1230399), which was statistically significant in both the discovery (p=0.004) and validation (p=0.028) analyses of femoral neck vBMD and explain 0.7% of the variation in the pooled sample (p=1x10-4). DKK2 and SOST can both act as antagonists of WNT signaling by binding to the LRP5/6 co-receptor and may both modify the activity of the WNT signaling pathway(35-37). Though additional investigation at both the population and functional level will be needed to confirm and characterize this relationship.

This study does not include a comprehensive set of tagging SNPs for each gene, but does provide information on a broad range of WNT pathway members. The 148 SNPs included in the analysis of gene-environment and gene-gene interactions were all associated with QCT measurements in the discovery sample and followed up on in the validation genotyping sample during previous main effects analyses. Assessing interactions between SNPs with statistically

significant main effects is a commonly employed strategy and has been used to study gene-gene interactions and BMD(38,39). However, our analysis of LRP5 and activity interactions revealed that SNPs with no main effects on BMD may have statistically significant interaction effects on vBMD.

While additional genotyping would have captured additional common variation in the WNT pathway genes, the additional statistical tests performed would have been amplified, especially in the gene-gene analysis that had almost 100 times the number of statistical tests as the gene-environment analysis. Utilization of dimension reduction techniques to reduce the number of statistical tests will need to be assessed to help address this problem. Hypotheses that incorporate functional evidence may also be helpful in assessing gene-environment and gene-gene interactions.

Much larger cohorts and replication samples will likely be needed to characterize gene-environment and gene-gene interaction effects on bone mineral density. However the LRP5-PASE analysis serves as a useful illustration that simply increasing sample size may not aid in identification of interactions. Kiel et al. reported an association with men but not women for this interaction and also showed evidence of different associations in different age groups(9). Replication samples that are comprised of different genders or that are ethnically diverse may complicate the biological relationships we hope to identify. Our analysis was unique in that we tested interactions in two, highly comparable samples.

Our follow-up analysis of the LRP5-PASE interaction was possible because both the current and previous analysis by Kiel et al. used a standardized and validated epidemiologic tool to measure physical activity(9). In the future, careful consideration of measurement tools for

environmental modifiers of genotype effects will be needed to ensure that comparisons can be drawn across populations and to help in collaborative efforts.

Although not without limitations, the current analyses demonstrate that even in a biological pathway that is known to be important in regulating BMD, significant interactions between genes and between genes and the environment may influence BMD variation. BMD is a highly heritable trait and genetic variants are thought to explain as much as 85% of the variance in this phenotype (40-44). To date, little of the variation in BMD has been explained by analyses that consider genetic variants alone (45) and animal models indicate that interactions may explain an sizeable portion of the variation in BMD(46). Advances in genotyping technology have allowed for higher throughput analysis of the relationship between genetic variants and BMD. However, the current analyses demonstrate that not only increasing the number of genetic markers tested, but also understanding genetic variation in the context of other genes and the environment will be needed to unravel the complex relationship between genetic variants and BMD.

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5.7 TABLES AND FIGURES

Table 5-1 Percent coverage and mean max r2 are calculated by comparing SNPs in the current analysis to SNPs with minor allele frequency greater than 5% in the CEU population from Phase 2 of the International HapMap Project.

Gene Name	Gene Symbol	Number of SNPs with Genotypes	Percent Coverage	Mean Max r^2
v-akt murine thymoma viral oncogene homolog 1	AKT1	2	12.5%	1.00
adenomatous polyposis coli	APC	8	75.0%	0.95
axin 1	AXIN1	9	38.7%	0.96
catenin (cadherin-associated protein), beta 1, 88kDa	CTNNB1	3	90.7%	0.98
dickkopf homolog 2 (Xenopus laevis)	DKK2	7	34.8%	0.99
dickkopf homolog 3 (Xenopus laevis)	DKK3	5	14.2%	0.90
dickkopf homolog 4 (Xenopus laevis)	DKK4	1	20.0%	1.00
dishevelled, dsh homolog 2 (Drosophila)	DVL2	5	71.4%	0.94
frizzled-related protein	FRZB	4	34.4%	0.97
frizzled homolog 1 (Drosophila)	FZD1	1	12.2%	0.99
frizzled homolog 6 (Drosophila)	FZD6	4	12.3%	0.99
frizzled homolog 7 (Drosophila)	FZD7	1	4.3%	0.98
glycogen synthase kinase 3 beta	GSK3B	5	28.7%	0.98
kringle containing transmembrane protein 1	KREMEN1	10	33.3%	0.96
low density lipoprotein receptor-related protein 5	LRP5	4	13.9%	0.94
low density lipoprotein receptor-related protein 6	LRP6	6	27.3%	0.98
secreted frizzled-related protein 1	SFRP1	2	7.5%	0.91
secreted frizzled-related protein 2	SFRP2	3	58.1%	0.98
secreted frizzled-related protein 3	SFRP4	3	27.7%	0.97
smoothened homolog (Drosophila)	SMO	4	47.6%	0.96
sclerosteosis	SOST	2	32.1%	0.92
v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	SRC	9	34.2%	0.96
WNT inhibitory factor 1	WIF1	2	13.4%	0.90
wingless-type MMTV integration site family, member 11	WNT11	5	26.2%	0.98
wingless-type MMTV integration site family, member 4	WNT4	3	15.6%	0.95
wingless-type MMTV integration site family, member 5A	WNT5A	7	25.0%	0.88
wingless-type MMTV integration site family, member 5B	WNT5B	7	36.8%	0.92
wingless-type MMTV integration site family, member 7A	WNT7A	5	75.0%	0.93

Table 5-2 Characteristics of Caucasian men included in the genotyping samples.

	Discovery Sample N=862	Validation Sample N=1156	Pooled Sample (Discovery + Validation) N=2018
Age	74 (5.8)	74 (6.0)	74 (5.9)
PASE Score	147 (60)	146 (68)	147 (65)
Weight (Kg)	85.3 (14.1)	82.9 (12.5)*	83.9 (13.3)
Height (cm)	173.5 (6.7)	174.9 (6.7)*	174.3 (6.7)
BMI	28.3 (4.1)	27.1 (3.6)*	27.6 (3.9)
Lumbar Spine vBMD (g/cc)	0.243 (0.041)	0.227 (0.038)*	0.234 (0.040)
Femoral Neck vBMD (g/cc)	0.304 (0.056)	0.274 (0.051)*	0.287 (0.056)

*** Statistically significant difference between means for the discovery and validation sample ($p<0.001$).**

Table 5-3 Replicated, consistent associations with femoral neck volumetric bone mineral density. Analyses are adjusted for age, clinic and population sub-structure.

SNP 1		SNP2		Discovery Sample		Validation Sample		Pooled Sample		
				β	p-value	β	p-value	β	p-value	r2
WNT5A	rs11918967	KREMEN1	rs134665	-0.008	0.037	-0.007	0.019	-0.007	0.002	0.004
WNT5A	rs11918967	LRP6	rs4763794	-0.008	0.045	-0.008	0.008	-0.007	0.003	0.004
WNT5A	rs11918967	LRP6	rs4477532	-0.008	0.049	-0.006	0.028	-0.006	0.008	0.003
DVL2	rs222836	LRP6	rs4763794	-0.008	0.034	-0.006	0.048	-0.006	0.007	0.003
DVL2	rs222837	AXIN1	rs370681	-0.011	0.005	-0.007	0.011	-0.009	0.002	0.006
LRP5	rs634918	DVL2	rs222851	-0.010	0.039	-0.011	0.003	-0.011	$4x10^{-4}$	0.006
LRP5	rs634918	DVL2	rs222850	-0.012	0.014	-0.011	0.003	-0.011	0.002	0.006
LRP5	rs634918	WNT4	rs7517829	0.010	0.039	0.008	0.031	0.008	0.005	0.004
FZD1	rs6465306	WNT11	rs7933711	0.009	0.048	0.011	0.005	0.011	$4x10^{-4}$	0.006
FZD6	rs827540	WNT11	rs583786	0.016	0.018	0.013	0.005	0.014	$4x10^{-4}$	0.006
FZD6	rs827528	WNT11	rs583786	0.016	0.019	0.013	0.007	0.014	$6x10^{-4}$	0.005
FZD6	rs951826	WNT11	rs583786	0.017	0.015	0.013	0.008	0.014	$5x10^{-4}$	0.006
DKK2	rs6827902	SOST	rs1230399	-0.015	0.004	-0.009	0.028	-0.012	1x10-4	0.007
SFRP2	rs12645134	SOST	rs1877632	0.011	0.040	0.008	0.043	0.010	0.004	0.004
SMO	rs6962740	AXIN1	rs540	0.010	0.040	0.009	0.012	0.010	$7x10^{-4}$	0.005

Table 5-4: Replicated, consistent associations with lumbar spine volumetric bone mineral density. Analyses are adjusted for age, clinic and population sub-structure.

SNP 1		SNP2		Discovery Sample		Validation Sample		Pooled Sample		
				β	p-value	β	p-value	β	p-value	r2
KREMEN1	rs132274	SRC	rs6094373	-0.008	0.014	-0.006	0.026	-0.007	$7x10^{-4}$	0.005
KREMEN1	rs132274	SRC	rs1547836	-0.008	0.017	-0.007	0.009	-0.007	$3x10^{-4}$	0.006
KREMEN1	rs132274	SRC	rs10470023	-0.007	0.033	-0.008	0.004			
KREMEN1	rs132274	SRC	rs6017944	-0.007	0.033	-0.008	0.004	-0.008	$3x10^{-4}$	0.006
DKK3	rs1472190	KREMEN1	rs738246	0.006	0.025	0.006	0.008	0.006	$4x10^{-4}$	0.006
FZD6	rs827528	SFRP1	rs10958671	-0.010	0.033	-0.010	0.004	-0.009	$8x10^{-4}$	0.005
FZD6	rs827540	SFRP1	rs10958671	-0.010	0.030	-0.008	0.018	-0.008	0.002	0.004
FZD7	rs1477031	KREMEN1	rs2294242	-0.012	0.011	-0.007	0.043	-0.009	$9x10^{-4}$	0.005
FZD7	rs1477031	WNT7A	rs2053101	0.007	0.026	0.006	0.019	0.006	0.005	0.004
DKK2	rs419764	WNT11	rs2004652	0.008	0.013	0.006	0.017	0.006	0.005	0.006
DKK4	rs3736649	DVL2	rs222850	0.014	0.016	0.009	0.048	0.001	0.002	0.004
DKK4	rs3736649	DVL2	rs222851	0.016	0.006	0.009	0.044	0.012	0.008	0.005
LRP5	rs312018	LRP6	rs12313200	0.009	0.023	0.008	0.011	0.008	$4x10^{-4}$	0.006

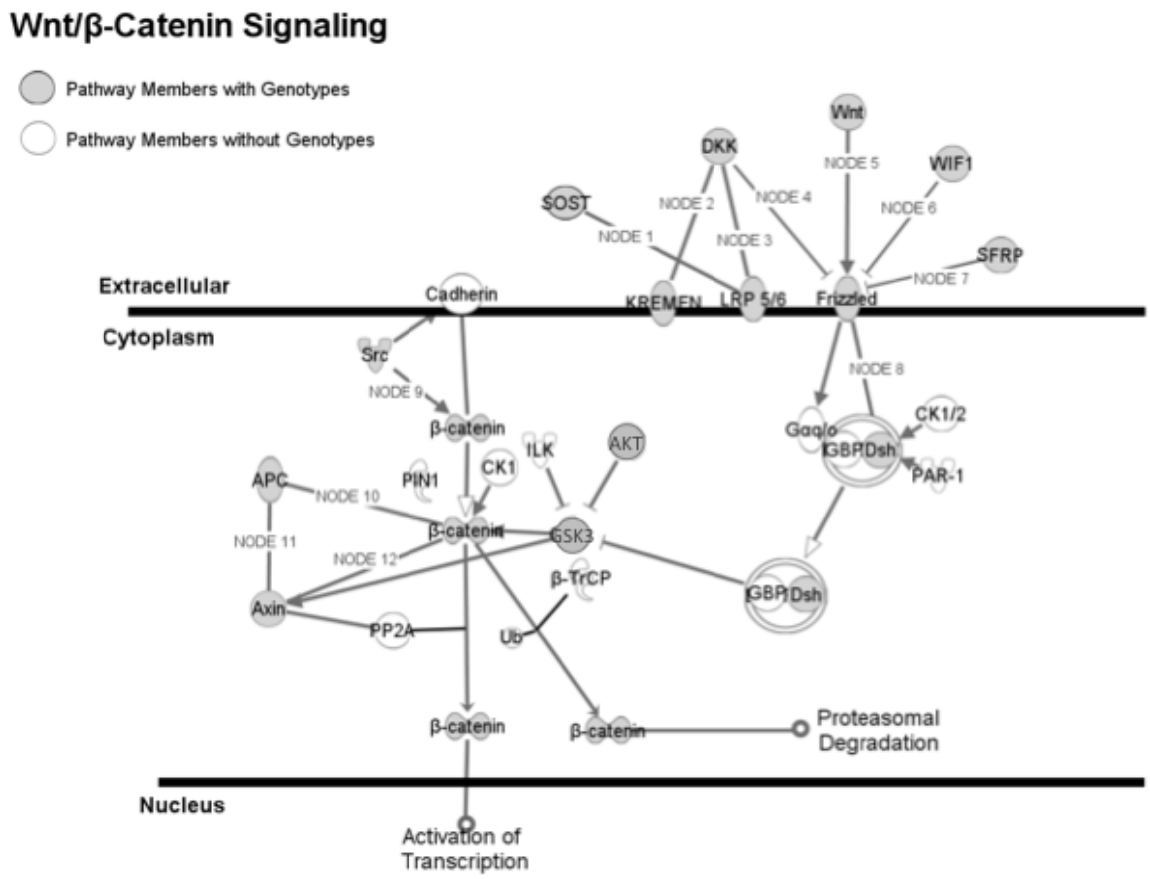

Figure 5-1 WNT Pathway Diagram.

Pathway members with genotypes included in this analysis are shown in grey.

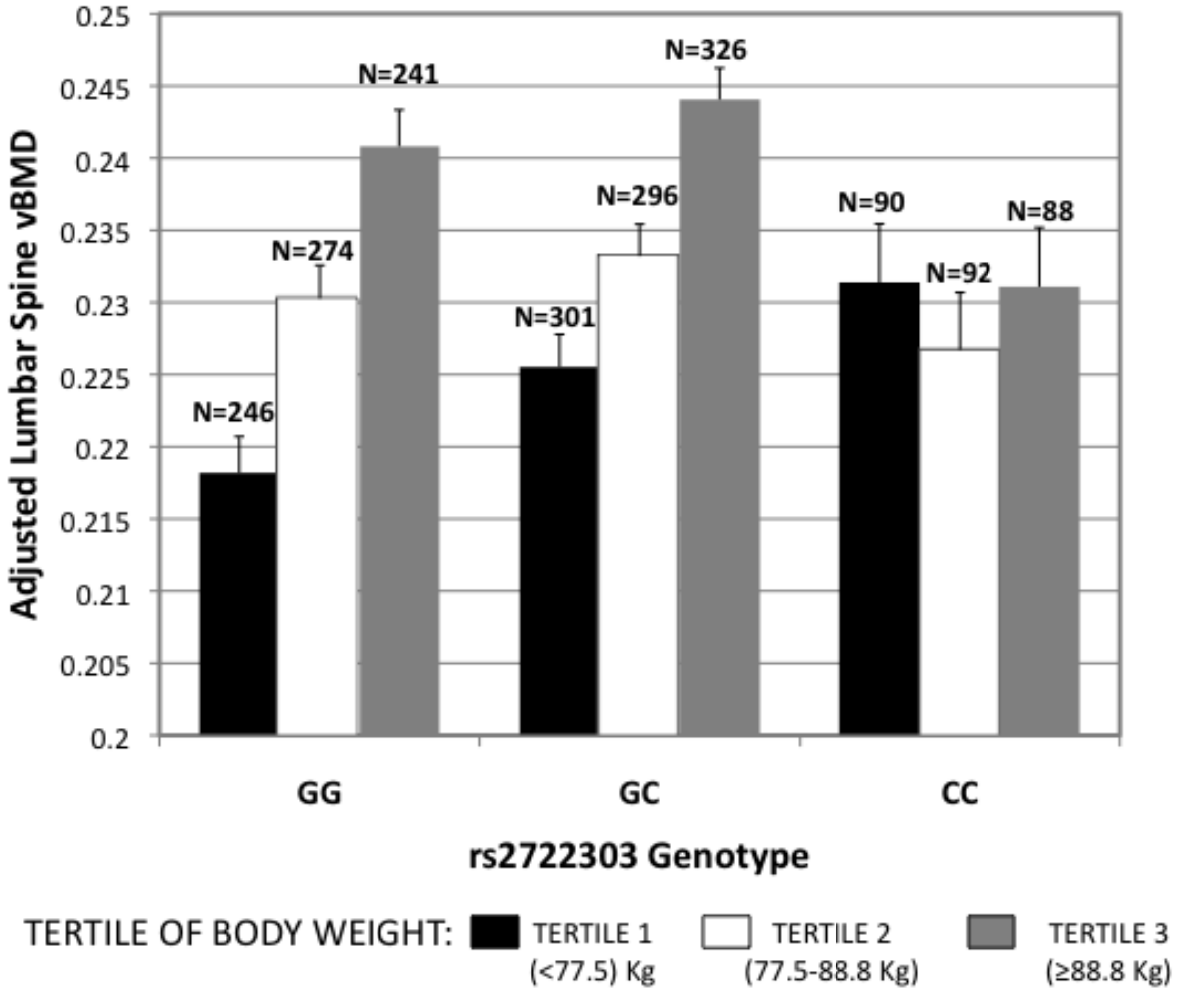

Figure 5-2 Significant interaction effect of rs2722303 in the SFRP4 gene and body weight on lumbar spine volumetric bone mineral density.

Means are adjusted for age, clinic and population sub-structure in the pooled sample. Tertiles of body weight are defined as follows: tertile 1 (black bars) <77.5 kg, tertile 2 (white bars) 77.5-88.8 kg, tertile 3 (grey bars) ≥88.8 kg.

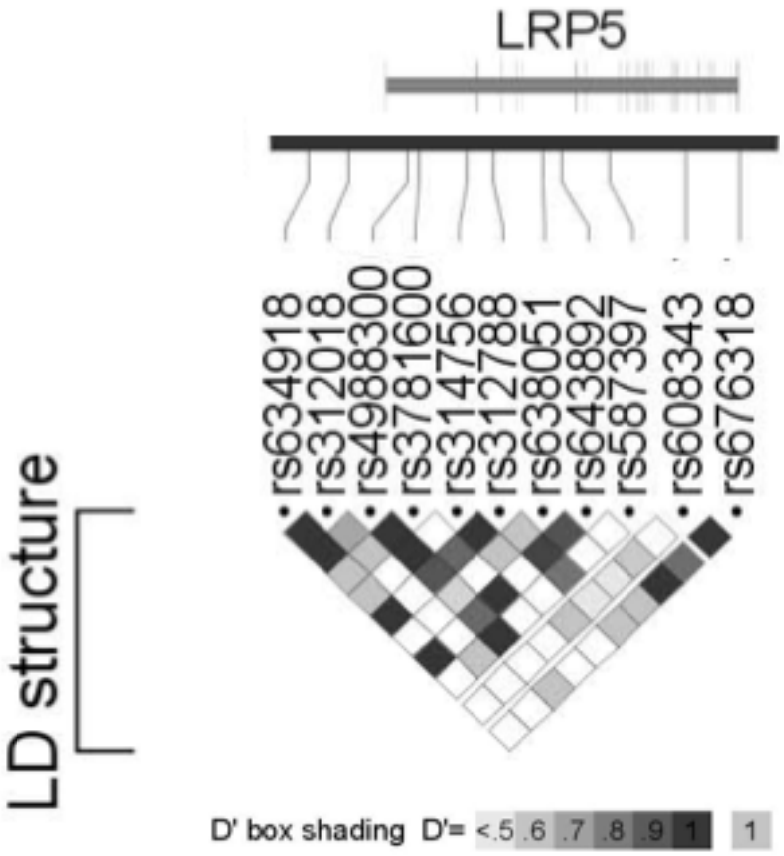

Figure 5-3 LRP5 SNP diagram and linkage disequilibrium (LD) pattern.

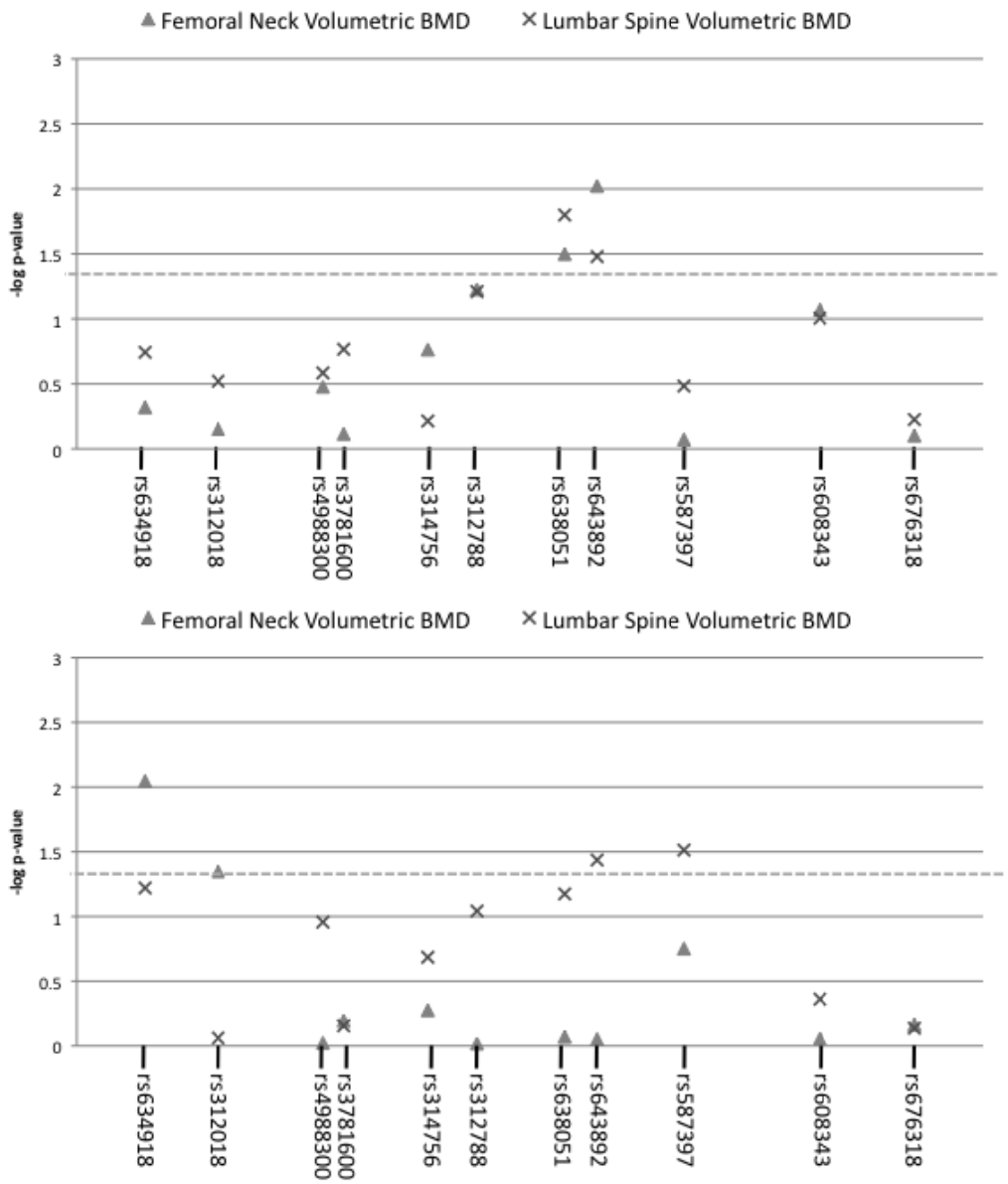

Figure 5-4 Interaction analysis for LRP5 SNPs and physical activity (PASE score) (part a) and body weight (part b).

Femoral neck volumetric bone mineral density (vBMD) is represented by triangle, lumbar spine vBMD is represented by x symbol. SNPs are placed on x-axis corresponding to chromosome position. Dotted line represents p=0.05.

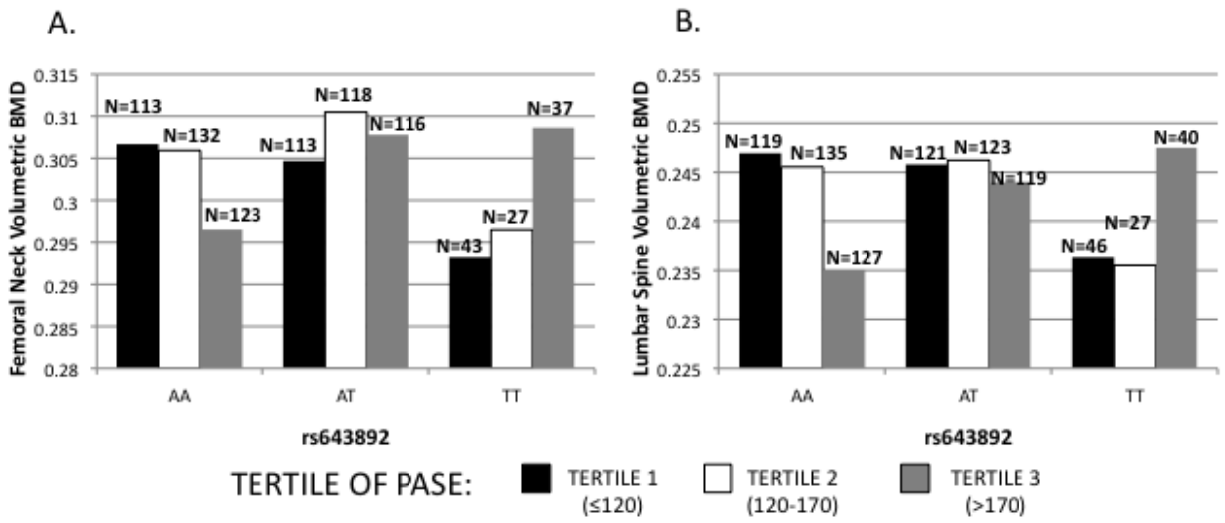

Figure 5-5 Significant interactions of rs643892 in the LRP5 gene and tertile of PASE score with femoral neck (part a) and lumbar spine volumteric BMD (part b) .

Volumetric bone mineral density is adjusted for age and clinic. Tertiles of PASE score are presented as follows: tertile 1 (black bars) ≤120, tertile 2 (white bars) 120-170, tertile 3 (grey bars) >170.

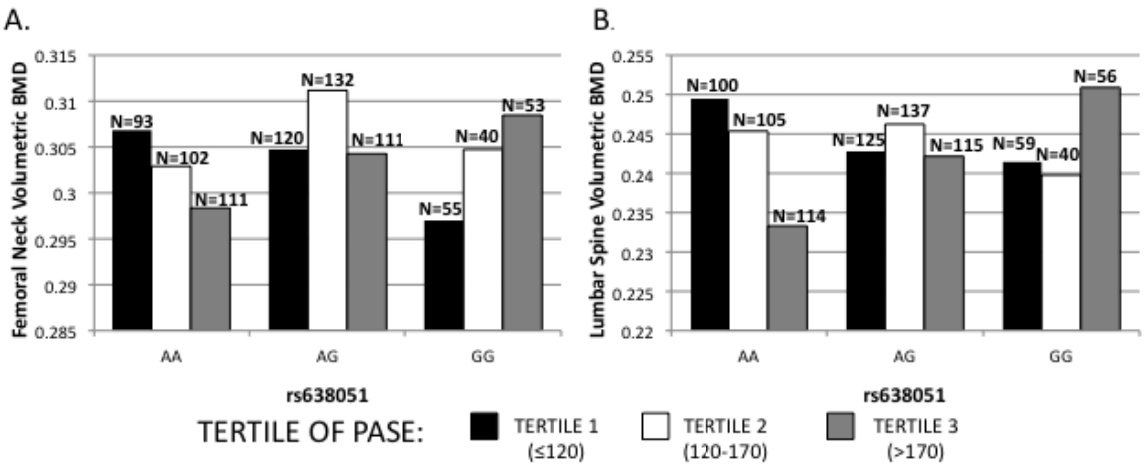

Figure 5-6 Significant interactions between rs638051 in the LRP5 gene and tertile of PASE score with femoral neck volumetric BMD (part a) and lumbar spine volumetric BMD (part b).

Volumetric bone mineral density is adjusted for age and clinic. Tertiles of PASE score are presented as follows: tertile 1 (black bars) ≤120, tertile 2 (white bars) 120-170, tertile 3 (grey bars) >170.

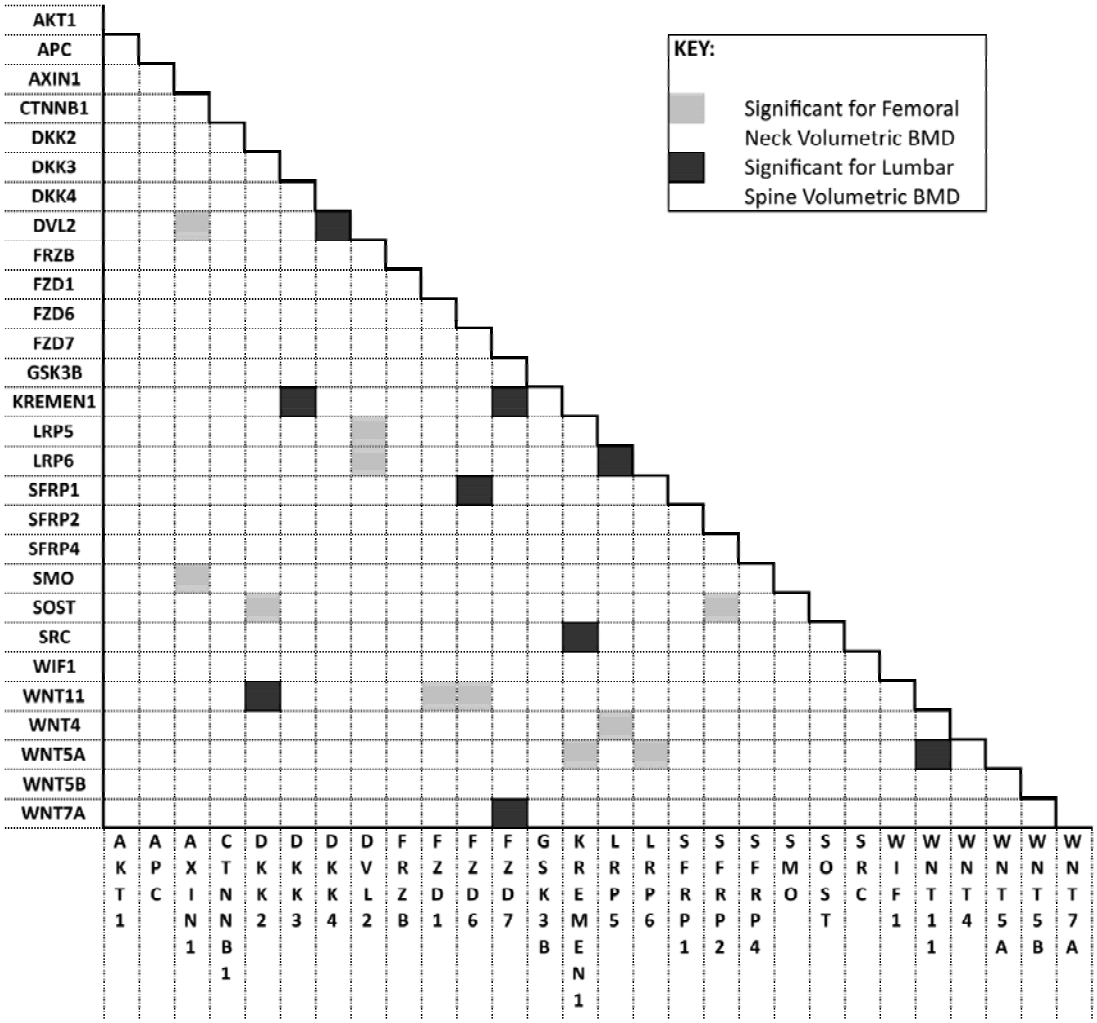

Figure 5-7 Summary of gene pairs with significant genotype-by-genotype interactions with femoral neck and lumbar spine volumetric bone mineral density.

6.0 GENERAL DISCUSSION

6.1 SUMMARY

Osteoporosis has been more carefully characterized in females since women bear much of the disease burden. However, osteoporosis is a substantial public health problem in males and with the aging trend in the male population, osteoporosis is predicted to become an even larger health problem in older men. BMD is used clinically to diagnose osteoporosis and research on the correlates of BMD has identified a number of demographic characteristics and environmental factors that influence BMD and osteoporosis risk. Genetics is also a substantial contributor to BMD and the subject of much inquiry. However, many questions remain to be explored.

One area of interest is the heterogeneity between the major clinically relevant skeletal sites like the femoral neck and lumbar spine. Though some genetic variants may influence BMD globally throughout the skeleton, others could be skeletal site specific. To examine this hypothesis we studied SNPs in 383 candidate genes for associations with integral vBMD at the femoral neck and lumbar spine in two samples of older Caucasian men. Statistically significant, consistent associations were identified for 8 SNPs in 6 genes for the femoral neck and for 13 SNPS in 7 genes for the lumbar spine. These genetic variants explained 1.7% of the phenotypic variation in femoral neck vBMD and 3.5% of the phenotypic variation in lumbar spine vBMD.

The majority of the genetic loci identified were unique to one skeletal site, but two gene regions (HOXA and APC) were associated with both femoral neck and lumbar spine vBMD.

Advances in bone imaging technology have allowed for images that can resolve both the trabecular and cortical bone compartments, butlittle is known about the genetic determinants of compartment specific BMD in humans. Statistical analysis between candidate genes and cortical vBMD at the femoral neck identified 7 SNPs in 5 genes that were consistently associated in both genotyping samples and explained 1.8% of the phenotypic variance. Consistent associations in 12 SNPs across 9 genes were identified for the femoral neck trabecular vBMD and these variants explained 4.0% of the phenotypic variation in trabecular vBMD. This analysis did not identify any SNPs that were associated with both cortical and trabecular vBMD. This, in combination with other findings, suggests the presence of genetic loci that are specific for each bone compartment[117-119, 121].

Much attention has been paid to understanding how individual genes and genetic variants influence BMD, but less is known about how genetic variants interact with each other and with environmental factors. With this in mind, an analysis of gene-environment and gene-gene interactions in 148 SNPs from 28 genes in the WNT/B-catenin signaling pathway, an influential pathway for BMD, was conducted. The WNT pathway is thought to be involved in mechanical loading[136] and mechanical loading is a major determinant of BMD. Thus, interactions between genetic variants in WNT pathway members and both weight and physical activity were explored. A statistically significant interaction between rs2722303 in the SFRP4 gene region and body weight was observed for lumbar spine vBMD. This interaction was significant in two genotyping samples and displayed a consistent pattern of association. Previous reports identified an interaction between SNPs in the LRP5 gene and physical activity[137], and our study was

able to replicate these findings. Finally, a number of statistically significant gene-gene interactions were identified between SNPs in different WNT pathway genes. However, multiple testing is a concern for this analysis and further research is needed to verify these findings.

Regression models for integral vBMD at the femoral neck and lumbar spine incorporating replicated main effects and replicated genotype-genotype and genotype-environment interactions were carried out to evaluate the amount of phenotypic variation explained by factors identified in this analysis. For the femoral neck, body weight, replicated genotype associations (Table 3-3) and replicated genotype-genotype interactions (Table 5-3) explained 7.9% of the phenotypic variation (adjusted r^2) in the pooled sample after adjusting for age, clinic, height and population sub-structure. For the lumbar spine, body weight, replicated genotype main effects (Table 3-4), the interaction between rs2722303 in SFRP4 and body weight, and replicated genotype-genotype interactions (Table 5-4) explained 9.3% of the phenotypic variation (adjusted r^2) in the pooled sample after adjusting for age, clinic, height and population sub-structure. Although body weight explains a portion of the increase (1% for femoral neck vBMD and 2.5% for lumbar spine vBMD), including interactions in the model explains a higher proportion of the phenotypic variance and demonstrates the importance evaluating genotype-genotype and genotype-environemtn interactions

6.2 STRENGTHS AND LIMIATIONS

This study was conducted in a large population of older men from the general population and employed a two-stage design that allowed for statistical associations to be replicated in a separate but comparable sample. The majority of genetics studies that have explored the genetics of

BMD have been in women making this study unique. However, this investigation was limited to studying older Caucasian men and the findings may not be generalizable to women, ethnic minorities or participants of younger ages.

The study is also distinct because it explores the genetics of vBMD measured by QCT and not areal BMD measured by DXA. QCT measurements of vBMD are not confounded by bone size and also allows for the trabecular and cortical bone compartments to be investigated[38]. In contrast, DXA measures integral areal BMD (trabecular and cortical BMD combined). However, measures of vBMD are not commonly made in population based studies making comparison of findings with other reports of genetic associations more difficult.

Although we employed a tagging SNP approach and also included potentially functional SNP variants, this study was designed using the first phase of the International Haplotype Map Project (HapMap). Publically available databases have improved since this study was designed and a more comprehensive assessment of genetic variation in the candidate genes is now possible. Additionally, this study was not designed to assess less common variants (MAF <5%) in the candidate genes that were investigated or other types of variants such as indels and structural variants. Finally, the functional significance of the associated SNPs was not tested and is currently unknown.

6.3 FUTURE DIRECTIONS

Extending these findings to populations that include females and ethnic minorities could confirm these associations and determine their generalizability. Although vBMD has many advantages it is a novel approach in population studies and is also costly. Therefore longitudinal measurements

using this technique are not currently available. The men in this association are older and likely actively loosing bone[98]. We cannot assess this active bone loss with the current, cross-sectional data, and may be detecting genetic associations with volumetric bone loss.

The cross-sectional nature of this analysis is also problematic when examining gene-environment interactions. Longitudinal covariate data may give important insights. For example, investigations of physical activity or weight change at midlife may be warranted.

Finally, additional genotyping may help refine and confirm associations. Fine-mapping of genetic associations may help pin-point causal variation that could be subsequently investigated in functional studies.

6.4 PUBLIC HEALTH SIGNIFICANCE

To better manage osteoporosis, improved prediction, prevention and treatment strategies are needed. Genetic data has the potential to help in all three of these aspects.

One of the essential public health services is to "evaluate effectiveness, accessibility, and quality of personal and population-based health services" which can be used to not only determine effectiveness of intervention programs but can also be used to retool existing intervention programs making them more effective[138]. Screening and clinical treatment protocols for osteoporosis are often reviewed and revised. One area of contention is when to screen and treat older men. A recent cost analysis identified two groups of men that should be screened using densitometry and treated if they have low BMD; men over age 65 with a previous fracture and those 80-85 years without a fracture history[139]. However, the authors contend that if treatment costs were to come down from $1,000 to $500, it would be cost-effective to

screen men >70 years of age regardless of fracture history[139]. Genetic testing is not inherently expensive (a proposed high-throughput method for testing for two hemochromatosis risk alleles is priced at only $8.00 per test) and costs are likely to come down as technology continues to improve[140]. Although individual genetic variants that explain a large portion of osteoporosis risk have not yet been identified, it is plausible that genetic testing of less detrimental variants could be incorporated with patient characteristics (e.g., body weight) and traditional clinical assessment tools (e.g., DXA) to aid in clinical decision making for osteoporosis in controversial risk groups (e.g., men >70 with no fracture history). In this context, genetics could aid in both the goals of prevention and prediction.

It is believed that pharmacogenomics, or the study of how genetic variation influences a patient's drug response, will be an essential tool in individualized medicine[141]. This is most commonly thought of in terms of drug development, and our improved understanding of the biology of osteoporosis is already driving such discovery[142]. However, improved understanding of how genetic variants interact with other genes and with their environment may be important in developing public health interventions in addition to treatment strategies. Examples of genetic variants with differential responses to vitamin D supplementation and hormone replacement therapy in osteoporosis already exist[126]. For example, our analysis of the interaction between WNT pathway members and body weight not only refines the biological relationship between mechanical loading and the WNT pathway, but could be a potential avenue for public health intervention. While obesity is a serious public health problem, low body weight in older populations is associated with frailty and morbidity[143]. Gene-environment interactions with body weight may have important public health Implications for lifestyle osteoporosis interventions.

Although translation from research to clinical practice has been slow, there is great potential for genetics as a public health tool in the fight against osteoporosis.

APPENDIX A

LISTING OF GENES INCLUDED IN THE ANALYSIS

Table A-1 Gene symbol, name and number of SNPs attempted and successfully genotyped for candidate genes.

Row Labels	Gene Name	Number of SNPs Attempted for Discovery Sample	Number of SNPs Passing QC for Discovery Sample	Percent Cover-age	mean max r^2
ACVR2B	Myostatin Receptor	4	4	72	0.984
ADIPOQ	Adiponectin (adipocyte, C1Q and collagen domain containing)	8	6	41	0.972
ADIPOR1	adiponectin receptor 1	7	7	72	0.975
ADIPOR2_WNT5BCluster*		24	20	54	0.948
ADIPOR2	Adiponectin receptor 2				
WNT5B	Wingless-type MMTV integration site family, member 5B				
AHSG	Alpha-2-HS-Glycoprotein	12	11	66	0.954
AKT1	v-akt murine thymoma viral oncogene homolog 1	8	6	87	0.964
AKT2	v-akt murine thymoma viral oncogene homolog 2	5	3	57	0.966
ALOX15	arachidonate 15-lipoxygenase	9	6	61	0.952
ALPL	Alkaline phosphatase, liver/bone/kidney	24	20	53	0.981
APC	adenomatosis polyposis coli	10	10	75	0.954
AR	Androgen Receptor	5	4	75	0.991
ARRB1	arrestin, beta 1	23	21	61	0.97

ARRB2	arrestin, beta 2	6	5	65	0.914
ATF2	activating transcription factor 2	10	10	89	0.968
ATF4	activating transcription factor 4 (tax-responsive enhancer element B67)	7	6	77	0.987
AXIN1	Axin 1	23	19	61	0.949
BAPX1	bagpipe homeobox homolog 1 (Drosophila)	6	5	48	0.939
BAX	BCL2-associated X protein	15	13	68	0.962
BCL2	B-cell CLL/lymphoma 2	44	39	66	0.97
BDNF	Brain Derived Neurotrophic Factor	11	10	78	0.957
BGLAP1	Bone Gamma Carboxyglutamate Protein 1 (aka Osteocalcin)	6	6	86	0.978
BGN	Biglycan	4	1	0	
BMP2	Bone Morphogenetic Protein 2	13	12	38	0.979
BMP2K	BMP2 inducible kinase	6	5	50	0.983
BMP3	Bone Morphogenetic Protein 3	10	8	83	0.952
BMP4	Bone Morphogenetic Protein 4	8	8	78	0.93
BMP7	bone morphogenetic protein 7 (osteogenic protein 1)	14	14	78	0.958
BMPR1A	BMP Receptor, Type IA	15	15	91	0.977
BMPR1B	BMP Receptor, Type IB	44	43	87	0.964
BMPR2	BMP Receptor, Type II	11	8	60	0.993
CALCA	Calcitonin	4	3	78	0.967
CALCR	Calcitonin Receptor	18	18	76	0.978
CASP2	caspase 2, apoptosis-related cysteine protease (neural precursor cell expressed, developmentally down-regulated 2)	8	5	84	0.996
CASP3	caspase 3, apoptosis-related cysteine protease	5	4	62	0.974
CASP8	caspase 8, apoptosis-related cysteine protease	7	7	75	0.945
CASR	Calcium Sensing Receptor	3	3	5	0.99
CBFB	core-binding factor, beta subunit	3	3	73	1
CCND1	cyclin D1	12	11	58	0.958
CDK6	cyclin-dependent kinase 6	20	18	81	0.942
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	19	13	53	0.955
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	16	12	27	0.974
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	6	5	71	0.994
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	8	8	94	0.98

CER1	Cerberus 1 homolog, cysteine knot superfamily (Xenopus laevis)	16	15	61	0.952
CHRD	chordin	9	9	33	1
CHRDL2	chordin-like 2	12	9	55	0.955
CHUK	conserved helix-loop-helix ubiquitous kinase (IKK1/IKKA)	6	6	82	0.94
CKTSF1B1	gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)	11	9	64	0.937
CLCN7	chloride channel 7	20	17	31	0.994
CNTF	ciliary neurotrophic factor	4	3	3	1
CNTFR	Ciliary Neurotrophic Factor Receptor - a	14	13	62	0.95
COL1A1	Type I Collagen, I alpha 1 Chain	8	7	48	0.948
COMT	Catechol-O-Methyltransferase	18	15	51	0.971
CRH	Corticotropin Releasing Hormone	4	4	96	1
CRHBP	Corticotropin releasing hormone binding protein	9	9	51	0.971
CRHR1	Corticotropin releasing hormone receptor 1	8	6	30	0.94
CRHR2	Corticotropin releasing hormone receptor 2	14	12	56	0.972
CSF1	Colony Stimulating Factor 1 (macrophage)	19	16	56	0.962
CSF1R	colony stimulating factor 1 receptor	17	15	43	0.937
CSF2	Colony Stimulating Factor 2 (granulocyte-macrophage)	14	11	85	0.958
CSF2RB	colony stimulating factor 2 receptor, beta	12	10	71	0.96
CSF3	colony stimulating factor 3 (granulocyte)	10	8	83	0.979
CSF3R	colony stimulating factor 3 receptor (granulocyte)	18	12	43	0.982
CTGF	connective tissue growth factor	13	13	55	0.957
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	5	4	93	0.98
CTSK	Cathepsin K (pycnodysostosis)	3	3	66	0.956
CYP11A1	Cholesterol Side-Chain Cleavage Enzyme	8	6	41	0.971
CYP11B1	Cytochrome P450, family 11, subfamily B, polypeptide 1	16	6	96	0.902
CYP17A1	17a-hydroxylase/17,20-lyase	11	11	94	0.984
CYP19A1	Aromatase	21	19	49	0.973
CYP1A cluster*		7	6	69	0.955
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1				
CYP1A2	cytochrome P450, family 1,				

		subfamily A, polypeptide 2				
CYP1B1		Cytochrome P450, Family 1, Subfamily B, Polypeptide 1	17	16	67	0.95
CYP21A2		Cytochrome P450, Family 21, Subfamily A, Polypeptide 2	5	3	0	
CYP24A1		Cytochrome P450, family 24, subfamily A, polypeptide 1	15	15	48	0.973
CYP27B1		Cytochrome P450, family 27, subfamily B, polypeptide 1	5	4	10	1
CYP3A4		Cytochrome P450, Family 3, Subfamily A, Polypeptide 4	4	3	66	1
DCN		Decorin	6	5	91	0.998
DKK1		dickkopf homolog 1 (Xenopus laevis)	7	7	62	0.933
DKK2		dickkopf homolog 2 (Xenopus laevis)	15	14	73	0.979
DKK3		dickkopf homolog 3 (Xenopus laevis)	16	14	35	0.94
DKK4		dickkopf homolog 4 (Xenopus laevis)	4	4	90	1
DLK1		delta-like 1 homolog (Drosophila)	10	8	60	0.975
DLL3		delta-like 3 (Drosophila)	8	7	83	0.987
DLX1_2Cluster*			14	14	8	1
	DLX1	distal-less homeobox 1				
	DLX2	distal-less homeobox 2				
DLX3		Distal-Less Homeo Box 3	7	7	75	0.984
DLX5		Distal-Less Homeo Box 5	7	6	50	0.96
DLX6		Distal-Less Homeo Box 6	6	4	17	0.93
DMP1		dentin matrix acidic phosphoprotein	8	8	72	0.98
DNTTIP2		deoxynucleotidyltransferase, terminal, interacting protein 2 (estrogen receptor binding protein)	7	7	95	0.98
DVL1		dishevelled, dsh homolog 1 (Drosophila)	3	3	0	
DVL2		dishevelled, dsh homolog 2 (Drosophila)	9	8	95	0.975
E2F1		E2F transcription factor 1	5	4	78	0.993
EGF		Epidermal Growth Factor (beta-urogastrone)	19	14	71	0.946
EGFR		Epidermal Growth Factor Receptor	43	40	58	0.958
EGR2		early growth response 2 (Krox-20 homolog, Drosophila)	7	7	54	0.98
EGR3		early growth response 3	9	9	41	0.983
EN1		engrailed homolog 1	12	11	75	0.992
ENPP1		ectonucleotide pyrophosphatase/phosphodiesterase 1	23	21	64	0.984
ESR1		Estrogen Receptor Alpha	36	33	70	0.975

ESR2	Estrogen Receptor Beta	11	11	72	0.956
ESRRA	estrogen-related receptor alpha	7	3	41	1
ETS2	v-ets erthroblastosis vius E26 oncogene homolog 2 (avian) [Homo sapiens]	19	18	61	0.978
EXT1	exostoses (multiple) 1, exostosin 1	18	17	51	0.965
EXT2	exostoses (multiple) 2, exostosin 2	14	13	74	0.958
FADD	Fas (TNFRSF6)-associated via death domain	4	4	55	0.962
FBXO32	F-Box Only Protein 32 (aka, Atrogin-1, MAFbx)	18	18	51	0.991
FGF1	Fibroblast Growth Factor 1	25	23	67	0.937
FGF18	Fibroblast growth factor 18	14	12	75	0.997
FGF2	Fibroblast Growth Factor 2	12	11	61	0.943
FGF23	fibroblast growth factor 23	10	8	38	0.934
FGF3	Fibroblast Growth Factor 3	11	7	29	0.961
FGFR1	Fibroblast Growth Factor 1 Receptor	13	12	84	0.956
FGFR2	Fibroblast Growth Factor 2 Receptor	63	55	59	0.967
FGFR3	Fibroblast Growth Factor 3 Receptor	5	4	66	1
FLT1	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	35	33	60	0.965
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	15	15	64	1
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	10	7	41	0.972
FOSL1	FOS-like antigen 1 (Fra1)	2	2	81	0.967
FOXC2	forkhead box C2 (MFH-1, mesenchyme forkhead 1)	12	10	58	0.949
FRZB	Frizzled-Related Protein	12	11	51	0.971
FST	Follistatin	9	8	36	0.983
FZD1	Frizzled homolog 1	6	6	65	0.977
FZD4	frizzled homolog 4 (Drosophila)	7	6	68	0.983
FZD5	frizzled homolog 5 (Drosophila)	6	5	40	0.973
FZD6	frizzled homolog 6	23	21	92	0.956
FZD7	frizzled homolog 7	9	9	52	0.993
GATA1	GATA binding protein 1	1	1	100	0.961
GATA2	GATA binding protein 2	9	8	62	0.989
GC	Vitamin D Binding Protein	11	10	61	0.969
GDF5	Growth differentiation factor 5 (cartilage-derived morphogenetic protein-1)	5	3	92	0.989
GDF8	Myostatin (Growth & Differentiation Factor 8)	5	5	56	0.961

GH1	Growth Hormone	7	4	76	0.922
GHR	Growth Hormone Receptor	18	15	81	0.968
GHRH	GH Releasing Hormone	5	4	27	0.992
GHRHR	GHRH Receptor	13	10	52	0.963
GHSR	growth hormone secretagogue receptor	9	9	69	0.958
GLI2	GLI-Kruppel family member GLI2	32	31	57	0.968
GLI3	GLI-Kruppel family member GLI3	34	32	44	0.96
GNRH1	Gonadotropin Releasing Hormone	5	4	79	0.994
GNRH2	Gonadotropin Releasing Hormone 2	9	8	45	0.983
GNRHR	Gonadotropin Releasing Hormone Receptor	9	9	83	0.98
GNRHR2	Gonadotropin Releasing Hormone Receptor 2	5	4	75	1
GPNMB	glycoprotein (transmembrane) nmb	7	6	89	0.939
GPR24	G protein-coupled receptor 24 (melanin-concentrating hormone receptor 1, MCHR1)	6	5	28	0.976
GPR54	G protein-coupled receptor 54	5	5	54	0.992
GSK3B	glycogen synthase kinase 3 beta	14	13	57	0.972
HDAC3	histone deacetylase 3	15	15	39	0.975
HES1	hairy and enhancer of split 1	11	9	61	0.983
HEY1	hairy/enhancer-of-split related with YRPW motif 1	10	10	51	0.977
HOXAcluster*		31	29	54	0.969
HOXA1	homeobox A1				
HOXA2	homeobox A2				
HOXA3	homeobox A3				
HOXA4	homeobox A4				
HOXA5	homeobox A5				
HOXA6	homeobox A6				
HOXA7	homeobox A7				
HOXA9	homeobox A9				
HOXA10	homeobox A10				
HOXA11	homeobox A11				
HOXA13	homeobox A13				
HOXDcluster*		28	26	78	0.962
HOXD1	homeobox D1				
HOXD3	homeobox D3				
HOXD4	homeobox D4				
HOXD8	homeobox D8				
HOXD9	homeobox D9				
HOXD10	homeobox D10				
HOXD11	homeobox D11				
HOXD12	homeobox D12				

	HOXD13	homeobox D13				
HSD11B1		Hydroxysteroid (11-Beta) Dehydrogenase 1	13	11	75	0.961
HSD17B1		17b-Hydroxysteroid Dehydrogenase I	6	3	16	0.922
HSD17B2		17b-Hydroxysteroid Dehydrogenase II	14	8	57	0.947
HSD17B3		17b-Hydroxysteroid Dehydrogenase III	18	13	77	0.954
HSD17B4		17b-Hydroxysteroid Dehydrogenase IV	13	12	76	0.956
HSD3B1		3b-Hydroxysteroid Dehydrogenase I	4	3	92	0.971
HSD3B2		3b-Hydroxysteroid Dehydrogenase II	5	4	85	0.958
IBSP		Integrin-Binding Sialoprotein (aka, Bone Sialoprotein, Bone Sialoprotein II)	13	10	57	0.952
ID1		inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	2	2	94	0.971
ID2		inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	5	5	90	0.992
ID3		inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	11	11	81	0.963
IFNAR1		interferon (alpha, beta and omega) receptor 1	8	7	53	0.947
IFNAR2		interferon (alpha, beta and omega) receptor 2	17	16	80	0.977
IFNB1		interferon, beta 1, fibroblast	6	6	56	0.994
IGF1		Insulin-Like Growth Factor I	14	13	72	0.961
IGF1R		IGF-I Receptor	42	40	42	0.96
IGF2		Insulin-like growth factor 2	8	6	50	0.981
IGF2R		IGF-II Receptor	38	30	75	0.981
IGFALS		IGFBP, Acid Labile Subunit	5	4	48	0.928
IGFBP1_3Cluster*			12	10	32	0.963
	IGFBP1	Insulin-like growth factor binding protein 1				
	IGFBP3	Insulin-like growth factor binding protein 3				
IGFBP2_5Cluster*			18	18	48	0.969
	IGFBP2	Insulin-like growth factor binding protein 2				
	IGFBP5	Insulin-like growth factor binding protein 5				
IGFBP4		IGF Binding Protein 4	9	8	81	0.961
IGFBP6		IGF Binding Protein 6	5	4	50	0.957
IHH		Indian hedgehog homolog	8	6	70	1
IKBKB		inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	7	6	52	1

IKBKG		inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	2	1	66	1
IL1Cluster		IL1 Cluster (IL1A and IL1B cluster)	20	17	85	0.972
	IL1A					
	IL1B					
IL1R1		IL1 Receptor I	19	18	77	0.968
IL1R2		IL1 Receptor II	12	12	79	0.983
IL1RN		IL1 Receptor Antagonist	14	12	61	0.953
IL6		Interleukin 6	12	11	50	0.954
IL6R		IL6 Receptor	10	10	69	0.975
IL6ST		IL6 Signal Transducer (aka, gp130)	6	6	93	0.99
IRAK1		IL1 Receptor-Associated Kinase 1	2	1	100	0.948
IRAK2_GHRLCluster*			34	30	57	0.955
	IRAK2	Interlukin-1 receptor-associated kinase 2				
	GHRL	ghrelin/obestatin prepropeptide				
IRAK3		Interleukin-1 receptor-associated kinase 3	11	11	60	0.952
IRS1		insulin receptor substrate 1	6	5	34	1
JAG1		jagged 1	16	12	52	0.957
KDR		kinase insert domain receptor (a type III receptor tyrosine kinase)	15	14	69	0.944
KL		klotho	17	16	60	0.962
KLF10		TGFB inducible early growth response	10	10	30	0.997
KREMEN1		kringle containing transmembrane protein 1	22	20	81	0.974
LCT		lactase	8	3	45	0.956
LEF1		lymphoid enhancer-binding factor 1	15	15	91	0.964
LEP		leptin (obesity homolog, mouse)	8	8	79	0.956
LEPR		leptin receptor	30	30	86	0.974
LHB		Luteinizing Hormone Beta	6	4	31	1
LHCGR		LH Receptor	29	27	53	0.963
LIF		Leukemia Inhibitory Factor	15	12	65	0.989
LIFR		Leukemia inhibitory factor receptor	15	13	70	0.902
LRP5		LDL-Receptor-Related Protein 5	15	11	51	0.966
LRP6		LDL-Receptor-Related Protein 6	19	19	79	0.968
LTBP1		Latent TGFB Binding Protein 1	51	45	24	0.952
LTBP2		Latent TGFB Binding Protein 2	21	20	70	0.955
LTBP3		Latent TGFB Binding Protein	5	5	61	0.982

		3				
MAP2K1		mitogen-activated protein kinase kinase 1	6	5	0	NA
MAP2K2		mitogen-activated protein kinase kinase 2	10	10	57	0.973
MAP3K1		mitogen-activated protein kinase kinase kinase 1	12	12	86	0.984
MAP3K14		mitogen-activated protein kinase kinase kinase 14 (NIK)	9	8	64	0.984
MAPK14		mitogen-activated protein kinase 14, p38, p38 MAP kinase	18	17	64	0.992
MAPK3		mitogen-activated protein kinase 3	4	4	53	0.968
MC2R		Melanocortin 2 receptor (adrenocorticotropic hormone)	4	4	50	0.941
MEF2A		MADS box transcription enhancer factor 2, polypeptide A (myocyte enhancer factor 2A)	10	9	41	0.96
MEF2B		MADS box transcription enhancer factor 2, polypeptide B (myocyte enhancer factor 2B)	9	7	52	0.974
MEF2C		MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)	12	12	64	0.978
MEF2D		MADS box transcription enhancer factor 2, polypeptide D (myocyte enhancer factor 2D)	15	14	86	0.977
MEPE		matrix, extracellular phosphoglycoprotein with ASARM motif (bone)	11	11	34	0.992
MGP		Matrix Gla Protein	5	5	96	0.947
MMP9		matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	13	10	80	0.978
MSX1		MSH Homeo Box Homolog 1	10	7	45	0.958
MSX2		MSH Homeo Box Homolog 2	11	10	75	0.958
MYD88		myeloid differentiation primary response gene (88)	6	4	60	0.992
MYFcluster*			11	11	87	0.983
	MYF5	myogenic factor 5				
	MYF6	myogenic factor 6				
MYOD1		MyoD (Myogenic Factor 3)	6	5	77	0.941
MYOG		Myogenin (Myogenic Factor 4)	2	2	80	0.986
NCOA1		nuclear receptor coactivator 1	13	11	74	0.96
NCOA2		nuclear receptor coactivator 2	21	20	86	0.988
NCOA3		Nuclear Receptor	17	16	88	0.985

	Coactivator 3				
NFATC1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	27	25	53	0.974
NFKB1	Nuclear Factor Kappa-B	15	12	86	0.937
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IKBA)	14	12	31	1
NFKBIB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	11	11	66	0.962
NOG	Noggin	4	4	56	0.985
NOTCH1	Notch 1	20	17	57	0.994
NOTCH2	Notch 2	4	3	73	0.925
NR3C1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	12	8	58	0.969
NR4A2	nuclear receptor subfamily 4, group A, member 2	5	5	78	0.976
NRF1	nuclear respiratory factor 1	19	19	82	0.97
NTF3	Neurotrophin 3	11	10	60	0.981
NTRK1	Neurotrophic tyrosine kinase, receptor, type 1	13	12	50	0.965
NTRK2	Neurotrophic Tyrosine Kinase, Receptor, Type 2	17	17	41	0.973
NTRK3	Neurotrophic tyrosine kinase, receptor, type 3	44	43	48	0.952
OGN	OSTEOGLYCIN	5	4	96	0.962
OSCAR	osteoclast-associated receptor	5	5	25	0.99
OSTF1	osteoclast stimulating factor 1	19	18	54	0.971
OSTM1	osteopetrosis associated transmembrane protein 1	6	6	91	0.92
OSTN	osteocrin	9	8	72	0.958
P2RX7	purinergic receptor P2X, ligand-gated ion channel, 7	17	17	65	0.974
PAX1	paired box gene 1	2	2	60	0.984
PAX3	Paired box gene 3 (Waardenburg syndrome 1)	24	23	58	0.978
PAX7	paired box gene 7	24	21	40	0.979
PAX9	paired box gene 9	10	9	67	0.986
PDLIM4	PDZ and LIM domain 4	13	12	75	0.978
PHOSPHO1	phosphatase, orphan 1	8	7	73	0.985
PIK3R5	phosphoinositide-3-kinase, regulatory subunit 5	12	12	70	0.979
POMC	Proopiomelanocortin (adrenocorticotropin)	6	6	75	0.989
POSTN	periostin, osteoblast specific factor	6	6	75	0.99
POU1F1	POU domain, class 1, transcription factor 1 (Pit1, growth hormone factor 1)	10	9	87	0.981
PPARA	peroxisome proliferative activated receptor, alpha	29	22	52	0.968

PPARD	peroxisome proliferative activated receptor, delta	12	11	77	0.946
PPARG	peroxisome proliferative activated receptor, gamma	19	17	83	0.97
PPARGC1A	peroxisome proliferative activated receptor, gamma, coactivator 1, alpha	33	32	52	0.97
PPP3R1	protein phosphatase 3 (formerly 2B), regulatory subunit B, 19kDa, alpha isoform (calcineurin B, type I)	6	5	94	0.997
PROP1	prophet of Pit1, paired-like homeodomain transcription factor	5	4	47	0.967
PTCH	patched	6	6	80	0.956
PTGER4	prostaglandin E receptor 4 (subtype EP4)	4	4	76	0.964
PTH	Parathyroid Hormone	3	3	84	0.97
PTHLH	parathyroid hormone-like hormone	13	12	84	0.965
PTHR1	PTH Receptor	10	9	75	0.96
PTN	pleiotrophin	26	24	66	0.965
RBL1	retinoblastoma-like 1 (p107)	6	6	96	0.982
RBL2	retinoblastoma-like 2 (p130)	9	5	67	0.99
RIPK1	receptor (TNFRSF)-interacting serine-threonine kinase 1	8	5	43	0.967
ROR2	receptor tyrosine kinase-like orphan receptor 2	28	26	73	0.96
RPS6KA3	ribosomal protein S6 kinase, 90kDa, polypeptide 3	7	3	50	0.931
RUNX1	runt-related transcription factor 1, CBFA2	60	56	60	0.953
RUNX2	Runt-Related Transcription Factor (aka Core Binding Factor Alpha 1)	17	15	55	0.952
RXRA	retinoid X receptor, alpha	16	15	75	0.963
RXRB	retinoid X receptor, beta	8	7	62	0.958
RXRG	retinoid X receptor, gamma	18	17	67	0.954
SFRP1	secreted frizzled-related protein 1	12	12	81	0.946
SFRP2	secreted frizzled-related protein 2	4	4	93	0.978
SFRP4	secreted frizzled-related protein 4	17	14	50	0.964
SHBG	Sex Hormone Binding Globulin	7	6	50	1
SHH	Sonic hedgehog homolog	17	15	66	0.988
SHOX2	short stature homeobox 2	6	5	94	0.997
SMAD1	SMAD, mothers against DPP homolog 1	10	10	93	0.952
SMAD2	SMAD, mothers against DPP homolog 2	11	7	80	0.89
SMAD3	SMAD, mothers against DPP	27	25	53	0.96

		homolog 3				
SMAD4		SMAD, mothers against DPP homolog 4	4	3	100	0.993
SMO		smoothened	10	9	52	1
SOST		Sclerosteosis (aka, Sclerostin)	12	7	32	0.919
SOX4		SRY (sex determining region Y)-box 4	3	3	75	1
SOX5		SRY (sex determining region Y)-box 5	39	38	19	0.958
SOX6		SRY (sex determining region Y)-box 6	27	27	52	0.981
SOX9		SRY (sex determining region Y)-box 9	6	5	82	0.91
SP7		Sp7 Transcription Factor (aka, Osterix, OSX)	3	3	64	0.934
SPARC		secreted protein, acidic, cysteine-rich (osteonectin)	12	12	78	0.96
SPP1		Secreted Phosphoprotein 1 (aka, Osteopontin, Bone Sialoprotein I)	9	8	81	0.96
SRC		v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian	20	20	81	0.98
STAR		Steroidogenic acute regulator	3	3	100	0.955
STAT1		signal transducer and activator of transcription 1, 91kDa	16	15	57	0.95
STS		Steroid sulfatase (STS)	8	3	75	0.979
TANK		TRAF family member-associated NFKB activator	13	11	86	0.985
TBX3		T-box 3	11	11	69	0.97
TBX2_4 Cluster*			25	22	70	0.961
	TBX2	T-box 2				
	TBX4	T-box 4				
TCF1		transcription factor 1, hepatic nuclear factor (HNF1), MODY3	11	9	52	0.981
TCF4		transcription factor 4	49	48	81	0.971
TCF7		transcription factor 7 (T-cell specific, HMG-box)	8	6	78	0.949
TCIRG1		T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 protein a isoform 3	5	4	68	0.99
TGFB1		Transforming Growth Factor b1	6	6	50	0.978
TGFB2		Transforming Growth Factor b2	18	17	75	0.964
TGFB3		Transforming Growth Factor b3	9	8	39	0.96
TGFBR1		TGFB Receptor I	8	7	88	0.966
TGFBR2		TGFB Receptor II	23	20	53	0.981
TGFBR3		TGFB Receptor III	42	39	68	0.97

THRA	Thyroid hormone receptor, alpha	8	7	52	0.996
THRB	Thyroid hormone receptor, beta	79	72	58	0.953
TLR4	toll-like receptor 4	11	10	65	0.97
TNFA	Tumor Necrosis Factor Alpha	7	6	46	0.956
TNFAIP3	tumor necrosis factor, alpha-induced protein 3 (A20)	7	5	54	0.97
TNFRSF10A	tumor necrosis factor receptor superfamily, member 10a (aka TRAILR)	18	17	79	0.959
TNFRSF11A	Receptor Activator of NF-kb	14	13	44	0.983
TNFRSF11B	Osteoprotegerin	14	14	53	0.991
TNFRSF1A	TNFA Receptor I	10	9	70	0.955
TNFRSF1B	TNFA Receptor II	25	24	69	0.991
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10 (aka TRAIL)	12	11	51	0.959
TNFSF11	Osteoprotegerin ligand	7	7	74	0.965
TOB1	transducer of ERBB2, 1	4	4	81	0.922
TRADD	TNFRSF1A-associated via death domain	4	4	77	0.978
TRAF1	TNF receptor-associated factor 1	6	5	90	0.99
TRAF2	TNF receptor-associated factor 2	6	3	75	0.946
TRAF6	TNF receptor-associated factor 6	13	8	96	0.972
TRH	Thyrotropin-releasing hormone	1	1	1	1
TRPV5	transient receptor potential cation channel, subfamily V, member 5	16	11	61	0.976
TSHR	Thyroid stimulating hormone receptor	30	28	72	0.96
TWIST	Twist homolog 1	5	4	57	0.965
TWIST2	Twist related protein 1	6	6	29	0.969
VDR	Vitamin D Receptor	22	20	59	0.963
VEGF	vascular endothelial growth factor	20	19	73	0.961
WDR5	WD repeat domain 5	1	1	0	NA
WIF1	WNT inhibitory factor 1	6	6	85	0.946
WISP1	WNT1 inducible signaling pathway protein 1	33	31	46	0.985
WISP2	WNT1 inducible signaling pathway protein 2	6	6	67	0.943
WISP3	WNT1 inducible signaling pathway protein 3	10	8	85	0.955
WNT10A	wingless-type MMTV integration site family, member 10A	7	6	69	0.957
WNT11	wingless-type MMTV integration site family, member 11	19	18	54	0.965

WNT3A		Wingless-type MMTV integration site family, member 3A	7	7	94	0.977
WNT4		Wingless-type MMTV integration site family, member 4	13	10	35	0.977
WNT5A		Wingless-type MMTV integration site family, member 5A	11	10	32	0.982
WNT7A		wingless-type MMTV integration site family, member 7A	14	11	44	0.934
WNT9A		wingless-type MMTV integration site family, member 9A (aka, WNT14)	5	5	70	0.987
WNTCluster*			11	10	100	1
	WNT1	wingless-type MMTV integration site family, member 1				
	WNT10B	wingless-type MMTV integration site family, member 10B				
ZFP67		zinc finger protein 67 homolog (c-krox, kruppel-related zinc finger protein hcKrox)	4	5	87	0.941
total			4608	4108		

APPENDIX B

PATHWAY-BASED ANALYSIS

Pathway based analysis was completed for genotyping done in the discovery sample for integral vBMD at the lumbar spine and femoral neck as well as for cortical and trabecular vBMD at the femoral neck. This analysis was conducted using a gene-set enrichment methodology described by Wang et. al [144]. Though designed for genome-wide association studies, this methodology was used to assess if particular biological pathways are associated with vBMD. Ingenuity Pathway Analysis software was used to define biological pathways (Table Appendix C-1). Nominal p-values, false-discovery rate and familywise error rate are presented for lumbar spine integral vBMD, femoral neck integral vBMD, femoral neck cortical vBMD, and femoral neck trabecular vBMD. No biological pathways were significant after adjusting for multiple comparisons but the NFKB pathway was nominally associated with lumbar spine vBMD ($p=0.049$) and the IGF pathway was nominally associated with cortical vBMD ($p=0.019$).

Table B-1 Pathway Definitions

Pathway Name	Pathway Member (gene symbol)
PPAR	ACDC, ADIPOR1, ADIPOR2, PPARA, PPARD, PPARG, PPARGC1A, MAPK14, MAP3K14, RXRA, MAP2K2, MAP2K1, MAPK3
FGF	FGF1, FGF18, FGF2, FGF23, FGF3, FGFR1, FGFR2, FGFR3, MAP3K1, MAPK14, MAP2K1, MAPK3
BMP	BMP2K, BMP3, BMP7, CHRD, FST, NOG, BMP2, BMP4, BMPR1A, BMPR1B, BMPR2
Vitamin D	CYP24A1, CYP27B1, GC, VDR, CEBPB, PTH, RXRB, RXRG, RXRA
Steroid Hormone	AR, COMT, CYP11A1, CYP11B1, CYP17A1, CYP19A1, CYP1A1, CYP1A2, CYP21A2, CYP3A4, ESR1, ESR2, ESRRA, GNRH1, GNRH2, GNRHR, GNRHR2, HSD17B1, HSD17B2, HSD17B3, HSD17B4, HSD3B1, HSD3B2, LHB, LHCGR, NCOA1, NCOA2, NCOA3, SHBG, STAR, STS
Glucocorticoid	CEBPA, CRH, CRHBP, CRHR1, CRHR2, CYP1B1, HSD11B1, MC2R, NR3C1, POMC, CEBPB
TGFB	ACVR2B, SMAD1, SMAD2, SMAD3, SMAD4, TGFB1, TGFB2, TGFB3, TGFBR1, TGFBR2, MAP2K2, MAP2K1, MAPK3
WNT	APC, AXIN1, CTNNB1, DKK1, DKK2, DKK3, DKK4, DVL1, DVL2, FRZB, FZD1, FZD4, FZD5, FZD6, FZD7, KREMEN1, LEF1, LRP5, LRP6, SFRP1, SFRP2, SFRP4, SMO, SOST, SOX5, SOX6, SRC, TCF4, WIF1, WISP1, WISP2, WISP3, WNT1, WNT10A, WNT10B, WNT11, WNT3A, WNT4, WNT5A, WNT5B, WNT7A, WNT9A, AKT1, AKT2, GSK3B
IGF	FOS, IGF1, IGF1R, IGF2, IGF2R, IGFALS, IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5, IGFBP6, IRS1, MAP2K2, MAP2K1, MAPK3
IL6	IL6, IL6R, IL6ST
Parathyroid	CALCA, CALCR, CASR, PTHLH, PTHR1, PTH
Thyroid	THRA, THRB, TRH, TSHR, RXRB, RXRG, RXRA
Cell Cycle	BAPX1, BAX, CASP2, CASP3, CASP8, CCND1, CDK6, CDKN1A, FADD, RBL1, RBL2, TANK, TNFRSF10A, TNFSF10, TRADD, CHUK, HDAC3, IKBKB, IKBKG, NFKB1, NFKBIA, NFKBIB, RIPK1, TNFA, TNFRSF1A, TNFRSF1B, TRAF2, GSK3B, MAP3K14, MAP2K2, MAP2K1, MAPK3
IFN	IFNAR1, IFNAR2, IFNB1, STAT1
EGF	EGR2, EGR3
Calcium	MEF2A, MEF2B, MEF2C, MEF2D, NFATC1, PPP3R1, ATF2, ATF4, HDAC3, MAPK3
NFKB	EGF, EGFR, GH1, GHR, IL1A, IL1B, IL1R1, IL1R2, IL1RN, IRAK1, IRAK3, MYD88, TLR4, TNFAIP3, TNFRSF11A, TNFRSF11B, TNFSF11, TRAF6, AKT1, AKT2, BMP2, BMP4, BMPR1A, BMPR1B, BMPR2, CHUK, IKBKB, IKBKG, NFKB1, NFKBIA, NFKBIB, RIPK1, TNFA, TNFRSF1A, TNFRSF1B, TRAF2, GSK3B, MAP3K14

Table B-2 Pathway-based analysis for lumbar spine integral volumetric bone mineral density.

Pathway	Nominal P-value	FDR P-value	FWER P-value
NFKB	0.049	0.373	0.319
IGF	0.058	0.504	0.648
WNT	0.139	0.500	0.794
TGFB	0.107	0.412	0.817
CellCycle	0.179	0.567	0.954
IL6	0.238	0.622	0.981
EGF	0.400	0.571	0.986
BMP	0.312	0.708	0.997
PPAR	0.405	0.837	0.999
Calcium	0.420	0.781	0.999
VitaminD	0.451	0.715	0.999
FGF	0.515	0.775	1.000
IFN	0.567	0.767	1.000
SteroidHormone	0.612	0.777	1.000
Thyroid	0.682	0.798	1.000
Parathyroid	0.968	1.000	1.000
Glucocorticoid	0.977	0.986	1.000

Table B-3 Pathway-based analysis of femoral neck integral volumetric bone mineral density.

Pathway	Nominal P-value	FDR P-value	FWER P-value
Calcium	0.054	0.542	0.413
WNT	0.242	1.000	0.991
IL6	0.362	1.000	0.998
FGF	0.297	1.000	1.000
IGF	0.412	1.000	1.000
TGFB	0.488	1.000	1.000
CellCycle	0.514	1.000	1.000
Parathyroid	0.799	1.000	1.000
PPAR	0.588	1.000	1.000
VitaminD	0.764	1.000	1.000
IFN	0.708	1.000	1.000
SteroidHormone	0.682	0.992	1.000
Glucocorticoid	0.883	0.983	1.000
BMP	0.899	0.987	1.000
Thyroid	0.898	0.992	1.000
NFKB	0.854	0.932	1.000
EGF	0.840	0.900	1.000

Table B-4 Pathway-based analysis of femoral neck cortical volumetric bone mineral density.

Pathway	Nominal P-value	FDR P-value	FWER P-value
IGF	0.019	0.176	0.163
VitaminD	0.025	0.398	0.538
IL6	0.444	1.000	0.996
WNT	0.318	1.000	0.997
TGFB	0.289	1.000	0.997
BMP	0.284	0.954	0.997
Calcium	0.335	0.936	0.998
FGF	0.355	0.838	0.999
Parathyroid	0.697	0.979	1.000
CellCycle	0.559	1.000	1.000
IFN	0.612	0.987	1.000
Glucocorticoid	0.787	0.911	1.000
NFKB	0.661	0.936	1.000
Thyroid	0.773	0.933	1.000
SteroidHormone	0.862	0.959	1.000
PPAR	0.894	0.944	1.000
EGF	0.970	0.943	1.000

Table B-5 Pathway-based analysis of femoral neck trabecular volumetric bone mineral density.

Pathway	Nominal P-value	FDR P-value	FWER P-value
WNT	0.078	0.636	0.479
NFKB	0.102	0.564	0.703
FGF	0.099	0.463	0.774
CellCycle	0.149	0.490	0.896
Calcium	0.192	0.691	0.986
IFN	0.242	0.736	0.996
EGF	0.482	0.651	0.996
IL6	0.367	0.578	0.997
PPAR	0.265	0.548	0.998
BMP	0.266	0.529	0.999
TGFB	0.357	0.592	1.000
VitaminD	0.387	0.602	1.000
IGF	0.425	0.583	1.000
Parathyroid	0.727	0.663	1.000
SteroidHormone	0.745	0.861	1.000
Thyroid	0.787	0.855	1.000
Glucocorticoid	0.879	0.972	1.000

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