GENETIC EPIDEMIOLOGY OF LONGITUDINAL CHANGE IN BONE MINERAL DENSITY IN MEXICAN AMERICANS: THE SAN ANTONIO FAMILY OSTEOPOROSIS STUDY

by

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Motivation: Bone mineral density (BMD), the principal determinant of bone strength and a risk factor for osteoporosis, is the net result of two processes: (i) the acquisition of peak BMD during young adulthood, and (ii) the subsequent rate of bone loss with age. While the genetics of peak BMD has been extensively studied, the specific genetic polymorphisms influencing peak BMD and the genetic contribution to bone loss are largely unknown. We investigated the extent to which genes influence 5-year change in BMD and searched for specific chromosomal regions influencing peak BMD and change in BMD in 1047 Mexican Americans from 34 large, multigenerational families.

Methods: BMD measurements of the hip, spine, and forearm were collected at baseline and follow-up (3-8 years later, mean = 5.6 years) by dual-energy x-ray absoptiometry, from which annual BMD change was calculated. Pedigree-based maximum likelihood methods modeling the variance decomposition of longitudinal and cross-sectional measurements of BMD were used to estimate heritability (h^2) and perform genome-wide linkage analysis (using a 7.6 cM genetic map) for BMD change and peak BMD. The effects of several environmental covariates, notably sex, age, weight, change in weight, and menopause, were simultaneously modeled.

Results: We determined that change in BMD varied over time and could be categorized into two heritable (h^2 = 31% to 44%) phases: early adult bone loss in participants <45 years of age and later bone loss in participants >45 years of age. A quantitative trait locus (QTL) influencing early bone loss was observed on chromosome 1q (LOD = 3.6) in the cohort <45 years; no specific chromosomal regions influencing change in BMD were observed in the cohort >45 years. By comparing cross-sectional genetic analyses at baseline and follow-up, we identified QTLs on chromosomes 6q and 13q with consistent effects on peak BMD of the hip and showed that QTLs influencing peak BMD did not overlap with QTLs influencing bone loss.

Public health significance: This work demonstrated the importance of genes in the etiology of osteoporosis, a growing public health problem. Understanding the genetic determinants of bone strength could lead to new biological targets for the treatment of osteoporosis, and/or the identification of persons at risk who would benefit from preventative interventions.

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PREFACE

I would like to express my deep appreciation to all of those who made this dissertation research possible. In particular, my sincere gratitude goes to the participants, investigators, collaborators, and funding sources of the San Antonio Family Osteoporosis Study for the opportunity to be a part of this outstanding research project, to my friends and family for their steadfast support, to my coworkers and lab mates for their advice and assistance, to my committee members for their thoughtful guidance, and to my committee chair and mentor for her tutelage, enthusiasm, and friendship. Thank you.

1.0 INTRODUCTION

1.1 PUBLIC HEALTH SIGNIFICANCE OF OSTEOPOROSIS

Osteoporosis is a skeletal condition of major public health significance, contributing toward risk of fragility fracture in women and men of all populations. The degenerative disorder and associated fragility fractures have devastating effects on health, resulting in substantial morbidity for all osteoporotic fractures, and increased mortality for hip and vertebral fractures (1). Characterized by low bone mass and deterioration of bone micro-architecture, osteoporosis affects 30% of postmenopausal women (2) and increases in prevalence to 70% for those women 80 years and older (3). The incidence of this condition and the corresponding financial burden of treatment are projected to increase as the American population ages; indeed, heath care expenditures for treatment of osteoporotic fractures were $14 billion in 1995 (4), and the estimated annual cost for treating hip fractures alone may exceed $250 billion in the next 40 years (5). Thus, osteoporosis and its related morbidities, mortality, and economic costs represent a serious public health problem.

Advanced age is the most important risk factor for osteoporosis, and is associated with decline in both bone mass and bone quality. However, many other risk factors for low bone mass have also been identified. A number of lifestyle and healthcare-related factors (enumerated in Table 1.1) are known to affect bone mass (3,6), and because many of these are largely

modifiable, they provide means through which preventative lifestyle changes can help reduce risk. On the other hand, many non-modifiable factors contributing to reduced bone mass also exist (3,6). Of these, family history and ancestry (which are partly indicative of underlying genetic risk factors) are of particular interest in this dissertation, which investigates the genetics of longitudinal bone loss and examines the changing role of genes on bone mass over the adult lifespan in Mexican Americans.

Table 1.1. Risk factors for low bone mass

Modifiable	Non-modifiable
smoking	age
alcohol consumption	female sex
low vitamin D	family history of osteoporosis
low calcium intake	prior fragility fracture
low physical activity	Caucasian or Asian ancestry
low weight / weight loss	low birth weigh
stress / depression	lactose intolerance
surgical or drug induced hypogonadism	early menopause
glucocorticoid therapy	numerous co-morbidities / malignancies

Substantial ethnic and geographic variation in bone mass (7-19), owing to differences in environmental and genetic risk factors, echoes the varying degree of burden of osteoporosis across populations. In the United States, for example, Mexican Americans have greater bone mass, on average, than non-Hispanic whites, and lower bone mass than African Americans (18). Likewise, considerable variation in osteoporosis, fragility fractures, and bone mass among populations in Latin American (e.g. Argentina, Brazil, Chile, Columbia, Cuba, Mexico, Peru, Venezuela and others) has been reported (19). The importance of environmental factors notwithstanding, the enormous variation in genetic ancestry among Latin American populations, resulting from varying degrees of admixture between European, Native American, African, and

Eastern Asian parental groups, is likely responsible for part of the variation in osteoporotic risk among populations from these countries. Moreover, rapid population expansion and increased longevity in many developing nations has led to the growing societal impact of osteoporosis (20). Despite differences in total burden among ethnically- and geographically-defined populations, osteoporosis embodies a growing concern, worldwide (19,20). Understanding the environmental and genetic determinants of bone mass, including those in under-represented populations such as Mexican Americans, may lead to better prevention, risk assessment, and treatment of osteoporosis.

1.2 COMPONENTS OF BONE STRENGTH: BONE MASS AND BONE QUALITY

Human bone is classified into two types distinguishable by porosity and microstructure: (i) cortical bone, which is the dense outer shell and shaft of long bones, and (ii) trabecular bone, which is porous bone comprised of a network of rod- and plate-like supports called trabeculae. While different anatomical sites (e.g. ultradistal radius and midpoint radius) have different proportions of cortical and trabecular bone, both of these types of bone are important in the study of osteoporosis.

The risk of bone fracture is related to two components of bone strength: bone mass and bone quality. The latter is a poorly defined and difficult-to-measure characteristic of bone, which encompasses microarchitecture and distribution of mineral within bone, as well as bone geometry and turnover. Quality of bone microarchitecture refers to how the trabeculae are 3-dimensionally interconnected in a structural lattice, specifically the abundance, thickness, and spacing of trabeculae, as well as cortical thickness and porosity. Mineralization is also an

important characteristic for quality bone as newly laid bone requires time to fully mineralize; thus rapid remodeling can result in temporarily decreased bone quality. Bone turnover, related to both microarchitecture and mineralization, refers to the dynamic processes of bone resorption and formation. Tipping the balance of these two processes leads to resorption cavities within the bone, resulting in loss of bone quality. In addition to the small-scale structural aspects of bone matrix, gross bone geometry is also a major component of bone quality. The magnitude of bone dimensions, including external diameter and cortical thickness, partially determine the mechanical strength of bone, and may be responsible for much of the difference in bone strength between men and women (21).

Interest in bone quality is increasing along with the development of imaging technology and capacity for computer modeling (21). As the technology to practically assess certain components of bone quality become available, studies investigating these aspects of bone strength may provide new insights into the pathogenesis, diagnosis, and treatment of osteoporosis. However, until recently, most epidemiological and genetic studies of osteoporosis have not specifically addressed measures of bone quality, due largely to the difficulty in assessing such characteristics. Instead, much work has focused on phenotypes related to bone mass, which are easily measured, and account for 70% or more of the variation in bone strength (3).

Bone mass is typically quantified in terms of bone mineral density (BMD), which is defined as mineral mass (g) distributed across a 2-dimensional projected area (cm^2) or 3-dimensional volume (cm^3). Measurable BMD (g/cm^2 or g/cm^3) is the net result of at least two processes: (i) the acquisition of peak bone mass during young adulthood, and (ii) the subsequent loss of bone with age. While BMD is a very accurate indicator of bone strength, there are some

limitations to using this measure. For one, BMD is frequently calculated from a 2-dimensional projected area, which does not fully represent bone size. Also, decline in BMD may be reflective of either loss of mineral mass or increase in bone area, which complicates interpretation of longitudinal changes in BMD. BMD, and specifically the acquisition of peak bone mass and its apparent decline over the lifespan has been the subject of a number of epidemiological studies.

1.3 GENETIC EPIDEMIOLOGY OF BONE MINERAL DENSITY

Because of its public health implications, a large body of work has sought to identify environmental and genetic factors related to osteoporosis, and specifically BMD. Numerous environmental risk factors for reduced BMD have been enumerated above. The high heritability of BMD (30% to 80% depending on skeletal site and population) has long been known and demonstrated in many populations (17,22-32). Mapping of specific quantitative trait loci (QTLs) implicated in BMD has been the goal of several studies. Table 1.2 enumerates the findings from the many linkage analysis scans seeking to identify chromosomal regions that affect variation in BMD (33-57). QTLs have been observed on nearly every chromosome, with some regions implicated in multiple studies, and others identified in a single study. Inconsistency of linkage results among these studies may be reflective of the complexity of regulation of BMD, including: (i) genetic heterogeneity among populations, (ii) differences in environmental risk factors, demographics, and their interactions with genes, and (iii) differences in elements of study design, such as sample size, family size, phenotype assessment, inclusion criteria, statistical power, and types I and II error.

Table 1.2. Chronological summary of previous linkage analyses for BMD (LOD > 2.0)

Reference	Population	Sample	Trait	Region	LOD
Johnson et al., 1997	Caucasian, US	ped = 1, n = 22	spine	11q12	5.74
Devoto et al., 1998	Caucasian, Canada	ped = 7, n = 149	femoral neck	1p36	2.29
			spine	2p23	2.25
			femoral neck	4qter	2.28
Koller et al., 1998	Mixed, US	sibs = 374, n = 835	femoral neck	11q12	3.50
			Ward's	11q12	2.84
Niu et al., 1999	Chinese, China	ped = 96, n = 218	wrist	2	2.15
Koller et al., 2000	Mixed women, US	ped = 286, n = 636	spine	1q21	3.11
			femoral neck	5q33	2.03
			femoral neck	6p11	2.13
Devoto et al., 2001	Caucasian, Canada	ped = 42, n = 254	femoral neck	1p36	3.02
Deng et al., 2002	Caucasian, US	ped = 53, n = 630	spine	4q13	3.08
			spine	12q24	2.17
			spine	13q33	2.43
			wrist	4q32	2.26
			femoral neck	10q26	2.29
Karasik et al., 2002	Caucasian, US	ped = 330, n = 1557	Ward's	8q24	2.13
			trochanter	21q22	2.39
			spine	12q24	2.08
			femoral neck	6p21	2.93
			trochanter	21qter	3.14
Styrkarsdottir et al., 2003	Caucasian, Iceland	ped = 207, n = 1323	hip + spine	6p	2.06
			hip + spine	17p	2.02
			spine	18p	2.12
			hip + spine	20p12	5.10
Wilson et al., 2003	Caucasian, UK	ped = 1094, n = 2188	whole body	1p36	2.38
			spine	3p22	2.72
			spine	16q12	2.11
		ped = 254, n = 587	spine	3.p22	2.07
Karasik et al., 2003	Caucasian, US	ped = 330, n = 1557	Ward's	8q24	2.13
			trochanter	21qter	3.14
		(men only)	femoral neck	4q34	2.06
		(women only)	trochanter	12q23	3.00
		(<60 years)	femoral neck	9q22	2.71
			spine	14q31	2.48
		(>60 years)	trochanter	17p13	2.03
			Ward's	17p13	2.31
Kammerer et al., 2003	Mexican Am., US	ped = 29, n = 664	wrist	4p	4.33
			trochanter	6q27	2.27
			femoral neck	2pter	3.98
			femoral neck	8p21	2.15
			trochanter	13q14	3.46
			femoral neck	13q14	2.51

Table 1.2. (continued)

Reference	Population	Sample	Trait	Region	LOD
Karasik et al., 2004	Caucasian, US	ped = 323, n = 1203	P.C. 2	1p36	2.10
			P.C. 1	1q21	2.10
			P.C. 1	8q24	2.20
			hip P.C.	16p13	2.00
Huang et al., 2004	Caucasian, US	ped = 79, n = 1896	NA	no LOD >2	
Shen et al., 2004	Caucasian, US	ped = 79, n = 1896	spine	7p14	2.64
			spine	11q23	3.13
			femoral neck	Xp11	2.15
			femoral neck	Xq27	2.57
			wrist	Xq21	2.54
			wrist	Xq26	2.87
			wrist	Xq27	4.30
		ped = 26, n = 1058	spine	20p12	2.33
			femoral neck	4q35	2.14
			femoral neck	11q12	2.04
			femoral neck	15q23	2.00
			femoral neck	20p12	2.33
			femoral neck	Xq26	2.23
			wrist	20p13	2.55
Econs et al., 2004	Caucasian women, US	ped = 134, n = 602	spine	1q21	4.30
Peacock et al., 2004	Caucasian women, US	peds = 381, n = 842	trochanter	14q	3.05
	African Am. women	peds = 132, n = 293	Ward's	14q	2.58
			princ. comp.	14q	2.60
			femoral neck	15q	3.43
			Ward's	15q	2.00
			princ. comp.	15q	2.10
Ralston et al., 2005	Caucasian, UK	ped = 715, n = 3691			
		(men only)	spine	3q25	2.43
			femoral neck	4q25	2.22
			femoral neck	7p14	2.28
			femoral neck	10q21	4.42
			femoral neck	16p13	2.52
		(women only)	femoral neck	4q25	2.55
			spine	18p11	2.83
			spine	20q13	3.20
			femoral neck	16q23	2.28
Devoto et al., 2005	Caucasian, Canada	ped = 40, n < 254	femoral neck	1p36	2.87
			spine	7p15	2.15

Table 1.2. (continued)

Reference	Population	Sample	Trait	Region	LOD
Peacock et al., 2005	Mixed men, US	ped = 225, n = 482	spine	1q	3.13
			spine	2p	3.16
			spine	14p	4.60
			femoral neck	2q	2.99
			femoral neck	18	2.37
			Ward's	21	2.78
Cheung et al., 2006	Chinese, China	ped = 306, n = 1459	spine	1q	2.36
Huang et al., 2006	Chinese, China	ped = 306, n = 1459	NA	NA	NA
Streetan et al., 2006	Caucasian (Amish), US	ped = 48, n = 964	radius	3q26	2.11
		(men only)	total hip	7q31	4.15
			total hip	12q24	2.6
			femoral neck	7q31	3.09
			femoral neck	18p11	2.07
			spine	21q22	3.36
		(women only)	femoral neck	1p36	2..02
			spine	1q21	2.11
		(<50 years)	radius	11q32	2.11
			femoral neck	14q23	2.16
		(>50 years)	spine	3p25	2.32
Xiao et al., 2006	Caucasian, US	ped = 451, n = 4126	wrist	2q32	2.23
			wrist	5q23	3.39
			wrist	7p15	2.15
			hip	2q32	2.11
			hip	3p14	2.29
			hip	3q27	2.55
			spine	3p25	2.09
		(men only)	wrist	1p36	2.81
			hip	7p12	3.01
			spine	13q12	2.5
			spine	13q33	2.96
		(women only)	wrist	3p25	3.31
			wrist	5q23	2.82
			wrist	6q24	3.05
			spine	3p25	2.61
			spine	15q13	4.49
Hsu et al., 2007	Chinese, China	sibs = 941, n = 3093	femoral neck	7p21	3.68
			total hip	2q24	3.65
			total hip	7p21	2.93
			total hip	16q21	3.14
			femoral neck	2q24	2.31
			femoral neck	7p21	3.68
			femoral neck	16q21	2.9
			spine	5q21	2.71

Table 1.2. (continued)

Reference	Population	Sample	Trait	Region	LOD
Hsu et al., 2007	Chinese, China	sibs = 941, n = 3093	whole body	2q31	2.71
			whole body	7p21	2.47
			whole body	16q22	2.52
		(women only)	spine	13q21	3.61
Wang et al., 2007	Caucasian, US	ped = 451, n = 4126	P.C. 1	2q32	3.35
Zhang et al., 2008	Caucasian, US	ped = 207, n = 2200	hip	12p12	2.79
			hip	22q13	2.16
			wrist	2p13	2.04
			wrist	10p14	2.31
			wrist	14q23	2.07
		(men only)	hip	15q26	2.93
			spine	7p21	2.1
		(women only)	wrist	2p13	2.64
			wrist	18q21	2.29
Willaert et al., 2008	Caucasian, Belgium	ped = 1, n = 34	spine	1p36	3.07

ped = number of pedigrees; sib = number of sibships: n = sample size; P.C. = principal component

Few of the QTLs identified via linkage analysis have led to the discovery of specific genetic variants affecting BMD. In particular, evidence of linkage on chromosome 11q12, which has been identified in several studies (41,46,49), is thought to be due to the low density lipoprotein receptor related protein 5 (LRP5) gene. Two genome-wide linkage scan meta-analyses have been performed (58,59) showing strongest evidence of linkage to chromosomes 1p13-q23, 9q31-33 (58), and 16pter-p12.3 (59).

In addition to linkage analysis scans, a number of association and candidate gene studies have also been performed, which implicate specific alleles in osteoporosis and BMD regulation. The majority of such studies have focused on genes with reasonable biological connections to bone regulation and structure, including collagenic and non-collagenic bone matrix proteins, adehesion molecules and ligands, calciotropic hormones/receptors, cytokines, growth factors/receptors, metabolic pathways, and sex hormones/receptors (60). Association and

candidate gene studies for BMD and osteoporosis have specifically looked for effects of the following (non-exhaustive) list of candidate genes: estrogen receptor (61-63), vitamin D receptor (62-68), collagen type 1 (63,67,69), parathyroid hormone receptor type 1 (63), interleukin 6 (63,70-73), tumor necrosis factor-alpha (74,75), calcitonin receptor (68,76,77), LRP5 (78), apolipoprotein E (79), osteocalcin (80), insulin-like growth factor (81), alpha 2HS glycoprotein (82), and the osteoclast-specific subunit of the vacuolar proton pump (83). Like those of the linkage scans, studies of candidate genes have produced inconsistent results, which again may be due to population differences between studies including genetic heterogeneity, ancestry, gender, age, menopausal status, and frequencies of polymorphisms. Additionally, the conflicting findings may be due to false positives caused by inappropriately chosen control groups, selection bias, or population substructure including admixture (60). Therefore more work is required to conclusively characterize the role of these gene candidates in the regulation of BMD.

Coinciding with the advent of affordable, high-density genotyping platforms for hundreds of thousands of single nucleotide polymorphisms (SNPs), genome-wide association studies for BMD are currently underway. One such study using the 100K SNP GeneChip marker set in the Framingham cohort found that the majority of the top genetic associations with BMD occurred for SNPs in genes that have not been previously studied with regard to bone strength phenotypes (84). This study is the first of undoubtedly many genome-wide scans which will test even greater numbers of SNPs in the search for new candidate loci that many affect osteoporosis.

1.4 EPIDEMIOLOGY OF LONGITUDINAL CHANGE IN BONE MINERAL DENSITY

Bone is continually being remodeled through the cycle of bone formation and resorption, thus changes in bone mass may result from an imbalance of these two processes of bone turnover. Observed BMD can therefore be conceptualized as the net result of (i) peak bone mass attainment (and maintenance) and (ii) subsequent loss of bone that occurs when resorption exceeds formation. Findings of cross-sectional studies of BMD may be reflective of either of these two processes, or both. However, many cross-sectional studies adjust analyses for age, thereby estimating peak BMD, and as a result, findings may pertain less to the process by which BMD changes over time, and more to the acquisition of peak BMD. Longitudinal studies of BMD, on the other hand, pertain primarily to the changes in BMD over time. In the existing body of literature, change in BMD has been quantified either as absolute or percent change over unit time.

Previous studies have demonstrated several environmental factors that affect BMD change, including baseline weight and interim change in weight, alcohol consumption, smoking, female gender, estrogen replacement therapy (85), and menopausal status (86). Mixed findings have been reported for the effects of exercise (85,87), calcium intake, and serum vitamin D level (87,88). Additionally, BMD change during the first year following a hip fracture is as much as 5 times that reported in the non-fractured population (88). The magnitude and timing of BMD change, and the effects of significant environmental factors, may differ among bone sites (85-87,89), and may differ between cortical and trabecular bone. Recent work suggests that substantial loss of trabecular bone may begin in the third decade (17,90-92), whereas loss of cortical bone may occur primarily in later life (17,91,92) in both sexes; however, the exact

timing of onset of age-related BMD change is largely uncertain. Estimates of rate of change derived from cross-sectional data are inconsistent with rate of change obtained from longitudinal data for forearm, hip (89), spine (86,89) and whole body (86) BMD. These results emphasize the need for longitudinal data to appropriately study the process of age-related decline in BMD.

Previous work comparing BMD change in older African American and Caucasian men (14), and women (8) show that Caucasians experience a greater decline in BMD over time than African Americans. These racial differences suggest that underlying genetic factors may influence change in BMD, which is discussed in the following section. Previously, no longitudinal studies have looked at BMD change in Mexican Americans.

1.5 GENETICS OF LONGITUDINAL CHANGE IN BONE MINERAL DENSITY

To date, very few studies have addressed the role of genetic factors in change in bone traits over time, and all of them have been limited to the same approach: estimating heritability of bone loss in twins/siblings. No linkage or genetic association studies have yet been performed for longitudinal change in bone phenotypes.

The National Heart, Lung and Blood Institute Twin Study, which included 25 monozygotic, and 21 dizygotic pairs of Caucasian male twins (45-55 years of age), was the first study to look at heritability of BMD change. Radial bone mass and BMD were measured at baseline, and again 16 years later. The findings of this study provide little evidence for heritability of change in BMD. Intraclass correlations for monozygotic twins (rMZ) and dizygotic twins (rDZ) were 0.52 and 0.49, respectively, for absolute change in radial bone mass, and 0.35 and 0.43, respectively, for absolute radial BMD change. While both rMZ and rDZ for

change in bone mass and BMD change were significantly greater than 0, they were similar in magnitude indicating that the correlations were not due to genetic factors. Heritability (h^2), which was approximated as twice the difference between rMZ and rDZ, was 0.06 and -0.15 (neither is statistically significant), respectively, for changes in bone mass and BMD (26).

This study design had several limitations that may have resulted in its failure to detect heritability of BMD change. Because the sample sizes were small, and less than 60% of the pairs measured at baseline were available at follow-up, this study was underpowered to detect modestly sized genetic effects. Also, the role of environmental covariates, which may have largely impacted the observed change in bone phenotypes, was not considered in calculations of either intraclass correlations or heritability. Adjusting for covariates is especially important for older twins who may have differentially accumulated environmental insults affecting bone phenotypes over the 16 years between measurements. Failure to do so may have masked genetic effects in an already underpowered study.

A second study in 21 mono- and 19 dizygotic twins (ages 24-75, both sexes) reported stronger evidence for the heritability of change in bone traits over 1.1 to 5.5 years. Estimates of rMZ and rDZ were 0.93 and 0.51, respectively, for annualized percent change in lumbar BMD, and 0.60 and 0.11, respectively, for annualized percent change in Ward's triangle (93). This study was the first to report statistically significant results despite small sample sizes and failure to consider environmental factors. The magnitude of the genetic effect on change in BMD demonstrated in this study may be inflated due to the study design—differences in the relative environments of mono- and dizygotic twins may have led to exaggerated heritability. Moreover, twins across a broad range of ages were included, muddling the interpretation of these results, since the rates of BMD change are known to vary with age.

Very recently, two larger studies in premenopausal (94) and peri- and postmenopausal (95) women have convincingly established that BMD change, at least in some populations and for some skeletal sites, is heritable. Hui et al., estimated that the heritability (h^2) of 5.7-year BMD change of the femoral neck in 388 Caucasian sisters (20-50 years of age from 178 sibships) was 0.35 to 0.40 (94). A complimentary study in 724 older female twins (177 mono- and 185 dizygotic pairs, ages 45 to 82 years) showed 4.9-year change in BMD was heritable for the spine ($h^2 = 0.38$), total forearm ($h^2 = 0.49$) and whole body ($h^2 = 0.44$), but not skeletal sites of the hip (95). These investigations were superior to the previous twin studies due to their larger sample sizes and adjustment for environmental covariates affecting BMD change. However, many questions remain, such as whether BMD change is heritable in men and non-Caucasian women, whether heritability or genetic correlation differs between younger and older cohorts, and whether genes influencing BMD change differ from those affecting peak BMD attainment or between skeletal sites.

In summary, few studies have been done to quantify or identify genetic factors affecting change in bone phenotypes. Certainly major barriers to this type of research are the logistics and expense of longitudinal, family-based study designs. Nevertheless, a growing body of knowledge indicates that change in bone over time may be due to genetic factors. More work is needed to clearly establish the heritability of BMD change, opening the way for investigation of more interesting questions regarding the genetics of BMD change, such as identifying linked chromosomal regions, associated polymorphisms, and gene × environment interactions.

1.6 THE SAN ANTONIO FAMILY OSTEOPOROSIS STUDY

The San Antonio Family Osteoporosis Study (SAFOS) was started in 1997 with the goal of studying the environmental and genetic determinants of bone strength-related phenotypes. As an ancillary to The San Antonio Heart Study (SAFHS) (96), recruitment for SAFOS was held in conjunction with the follow-up phase of SAFHS. Probands of Mexican American ancestry were identified in a low-income neighborhood of San Antonio, and all first-, second-, and third-degree relatives of probands and spouses were invited to participate irrespective of medical history. Inclusion criteria for probands stipulated only that they be 40 to 60 years of age with large extended families in the San Antonio area. Body composition data measured by dual-energy x-ray absorptiometry (DXA) was collected for 895 unselected Mexican Americans from 34 multigenerational families. An example pedigree is shown in Figure 1.1. Eleven individuals were excluded from the study due to use of corticosteroids and aberrant DXA measurements, thus the total sample size for this study is n = 884. Data on numerous demographic, anthropologic, medical, reproductive, and lifestyle traits were collected, as well as DNA samples used for the genotyping of 460 highly-polymorphic microsatellite markers. To date, the SAFOS has yielded numerous insights into the genetic causes and environmental correlates of variation in BMD.

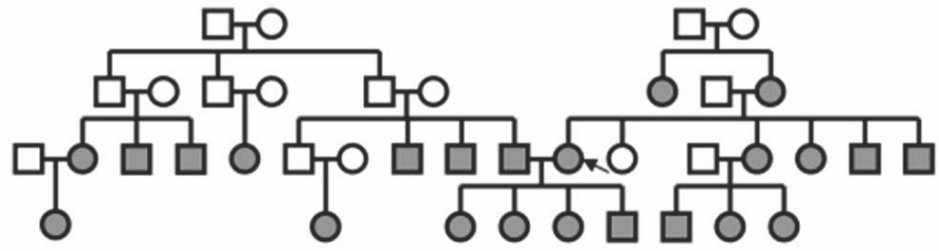

Figure 1.1. Sample pedigree. Arrow indicates proband. Data are available on shaded individuals

The co-morbidity of osteoporosis and atherosclerosis was explored by Kammerer et al. (97), in which the association between BMD and intimal medial thickness (IMT) of the carotid artery (i.e. a measure of pre-clinical atherosclerosis) was reported in female SAFOS participants ($p < 0.05$ for hip, spine, and forearm skeletal sites). Associations were also observed for a polymorphism of the vitamin D receptor gene (VDR) with both IMT and BMD (of the spine and forearm, but not hip), though BMD did not account for the relationship between IMT and VDR (97). This work was followed-up by Shaffer et al., who reported the association between IMT and forearm BMD in men, and showed that serum lipid traits and markers of inflammation (i.e. known risk factors for atherosclerosis) did not mediate the relationship between BMD and IMT in men and women (98).

Heritability and effects of environmental determinants of BMD were previously assessed separately for men and women in the SAFOS sample. Mitchell et al. (30) estimated that the heritable component of common variation in BMD was 0.22 to 0.62 in men, and 0.20 to 0.56 in women for skeletal sites at the hip, spine, and forearm. The total proportion of BMD variation explained by environmental correlates (i.e. age, age^2, diabetes, education, smoking, alcohol consumption, physical activity, dietary calcium, supplemental calcium intake, body mass index, menopause, oral contraception, hormone replacement therapy, parity, breastfeeding) was 0.05 to 0.47 in men and 0.26 to 0.46 in women (30).

Motivated by the high heritability of BMD phenotypes in this population, linkage analysis was undertaken in a subset ($n = 664$, approximately 75% of the total sample for whom genotype data were available) of the SAFOS population using 416 microsatellite markers (9.5 cM mean resolution) to identify specific genomic regions influencing BMD. Kammerer et al. (42), reported significant (99) QTLs on chromosome 4p for forearm BMD (logarithm of the

odds, LOD = 4.33), chromosome 2p for femoral neck BMD in men (LOD = 3.98), and chromosome 13q for trochanter BMD in men (LOD = 3.46). Suggestive evidence of linkage was also observed on chromosome 6q for trochanter BMD (LOD = 2.27), chromosomes 7q and 12q for forearm BMD (LOD = 2.24 for both), chromosomes 8p and 13q for femoral neck BMD in men (LOD = 2.15 and 2.51, respectively), and chromosome 20 for forearm BMD in women (LOD = 2.18) (42).

Investigation of the strong QTL on chromosome 4p for forearm BMD was continued by Chaney et al. (100) via genetic association testing of promising positional candidate genes in the region. Polymorphisms in one such gene, fibroblast growth factor binding protein 1 (FGFBP1), which had not previously been studied with regard to osteoporosis, showed strong association with BMD. These findings were replicated in an independent population (Amish), and functional studies revealed that three polymorphisms in FGFBP1 were associated with decreased gene expression *in vitro*. However, polymorphisms in FGFBP1 did not account for the linkage signal, therefore high density SNP genotyping covering the remaining genes in this region was carried out to search for other candidate genes. Polymorphisms in four additional genes in this region showed association to BMD, but they did not account for the linkage signal in the SAFOS population. Because genotype coverage of this region was not complete, one or more un-typed polymorphisms may account for the QTL on chromosome 4p (100).

The follow-up phase of SAFOS began in 2003 with the reenrollment of 724 (81%) participants from the original sample in addition to 163 new participants from the SAFOS families (total follow-up sample, n = 887). Body composition and covariate measurements were again collected (approximately 5 years after initial measurements) and genotyping for all participants was completed.

1.7 OVERVIEW OF ANALYTICAL METHODS

The principal methodologies used in this dissertation research are described below. These methods have been used to investigate the questions enumerated in Section 1.8.

1.7.1 Cross-sectional analysis of longitudinally-assessed measures

A major focus of this dissertation was to explore the genetic and environmental determinants of longitudinally-assessed change in BMD, which was calculated from BMD measured at two time-points. While we utilized a longitudinal study design to collect data on BMD change over time, we did not employ longitudinal analytical methods in this dissertation. Instead, we analyzed BMD change as a single metric, thus, this project is essentially a cross-sectional investigation of a longitudinally-assessed trait. In the following chapters we use the terms "cross-sectional" and "longitudinal" to reflect how the phenotype of interest was *measured* (e.g. baseline BMD is a cross-sectional measurement whereas BMD change is a longitudinal measurement), rather than how the data were analyzed.

1.7.2 Metric for BMD change

We calculated and performed analyses on two metrics of BMD change: (i) annualized absolute change in BMD (mg/cm^2/year) defined as the difference between follow-up and baseline BMD measurements divided by the exact elapsed time between measurements and (ii) annualized change in standardized BMD (%SD/year) defined as 100% times the difference between independently standardized (mean = 0, SD = 1) measurements of follow-up and baseline BMD

divided by the exact elapsed time between measurements. The latter metric, which was adopted to prevent possible bias due to differences in densitometry at the two time points, retains only information about the variance of BMD change (not the magnitude). Results of analyses for these two metrics showed excellent agreement; therefore we present results for only one of them. In Chapter 3 we show results for annualized absolute change in BMD and, in response to reviewers' concerns, state that results for annualized change in standardized BMD were nearly identical. In Chapter 4 we show results for annualized change in standardized BMD.

Another metric commonly used to quantify change in BMD in other studies is yearly percent change in BMD (%/year) defined as 100% times the annualized absolute change divided by baseline BMD. We do not present results of analyses for this metric. However, results from preliminary analyses of percent BMD change on data from a subset of 609 individuals (conducted while follow-up data collection was still underway) were very similar to those for annualized absolute change.

1.7.3 Covariate selection

The effects of a large number of environmental and endogenous covariates were originally considered in this project (Table 1.3). As described below, different procedures were used to select covariates for analyses of longitudinally-assessed BMD change in (i) older and (ii) younger individuals, and (iii) cross-sectional BMD at baseline and follow-up. These differing procedures resulted from the availability and comparability of covariate data among the different groups, however, after identifying a set of possibly-significant covariates, all covariates were assessed within the variance component framework (described in Section 1.7.4) allowing for non-independence of observations.

Table 1.3. Environmental and endogenous covariates considered in this study

demographic	anthropometric	reproductive	medical	lifestyle
sex	height	baseline menopause	diabetes	physical activity
age	change in height	follow-up menopause	hypertension	smoking
age^2	weight	interim menopause		alcohol consumption
sex × age	change in weight	oral contraceptives		calcium supplement
sex × age^2	body mass index	parity		calcium intake
	change in body mass index	months of breastfeeding		education
	baseline BMD	hormone replacement therapy		

Environmental determinants of BMD change were initially assessed in a sub-sample of 609 individuals while follow-up data collection was still underway. Bi-directional stepwise multiple regression analysis, while ignoring the familial dependency of the observations, was used to determine suites of covariates with possible effects on BMD change based on Akaike Information Criterion. Such analyses may result in inflated significance of covariate effects. Based on these preliminary analyses, several possible covariates were excluded from future consideration, such as parity, oral contraceptives, breastfeeding, hormone replacement therapy, hypertension, physical activity, smoking, alcohol consumption, use of calcium supplements, calcium intake, and education. The remaining covariates, including sex, age, age^2, sex × age, sex × age^2, baseline BMD, weight, change in weight, height, change in height, body mass index, change in body mass index, baseline menopause, and follow-up menopause, were re-evaluated via bi-directional stepwise multiple regression (again, while ignoring family structure) in the total sample after follow-up data collection was completed. Because weight and body mass index are highly correlated, these covariates were included one-at-a-time in regression models and whichever explained more phenotypic variation was retained.

For simplicity, covariates showing effects on BMD change in stepwise regression analyses in older individuals for one or more skeletal sites were forced into the final variance

components models presented in Chapter 3. This suite of covariates includes sex, age, age^2, sex × age, sex × age^2, baseline BMD, weight, change in weight, change in height, baseline menopause, and follow-up menopause.

A different covariate selection procedure was used for BMD change in younger individuals (Chapter 4). Covariates identified via stepwise regression in the total sample were tested within the variance components framework (which considers the pedigree structure of the data) and retained if statistically significant at a liberal threshold of $\alpha = 0.1$ on a skeletal site-specific basis.

Another covariate selection procedure was used in analysis of baseline and follow-up BMD (Chapters 5 and 6). Environmental determinants of baseline BMD have been previously reported (30). However, not all covariates were reassessed at follow-up. Because a major focus of this project was to compare cross-sectional analyses of baseline and follow-up BMD, we limited our possible suites of covariates to those assessed at both time points (i.e. sex, age, age^2, sex × age, sex × age^2, body mass index, menopause, oral contraceptives, diabetes, smoking, alcohol consumption, and supplemental calcium intake). Covariates identified via stepwise regression in the baseline and follow-up samples were tested within the variance components framework and retained at the threshold of $\alpha = 0.1$ on a skeletal site-specific basis.

1.7.4 Variance components analysis

In this dissertation, the environmental and genetic determinants of BMD and change in BMD were modeled within a variance components framework using the Sequential Oligogenic Linkage Analysis Routines (SOLAR) software (101). In brief, this methodology partitions the

total phenotypic variance into environmental, genetic (i.e. polygenic and QTL), and error components. The variance model takes the general form:

$$\sigma_T^2 = \sigma_E^2 + \sigma_G^2 + \sigma_e^2,$$

where σ_T^2 is the total phenotypic variance, σ_E^2 is the environmental component, σ_G^2 is the genetic component, and σ_e^2 is the error component. In this model, the environmental component encompasses the variance due to the effects of measured covariates. The genetic component may be further partitioned into two components: (i) a QTL component, σ_m^2, which is based on genetic sharing identity-by-decent (IBD) at an arbitrary chromosomal position as estimated from genotyped markers via a Markov Chain Monte Carlo algorithm implemented in Loki (102), and (ii) a residual heritable component, σ_g^2, also called the polygenic component, which is based on the expected genetic sharing between relatives and comprises the variance attributable to all additive genetic loci excluding that due to the QTL component (if any). The polygenic component is used to estimate the two measures of phenotype heritability considered in this dissertation: (i) the narrow-sense heritability, h^2, which is calculated as the ratio of polygenic component to total phenotypic variance:

$$h^2 = \frac{\sigma_g^2}{\sigma_T^2},$$

and (ii) the residual heritability, h^2_r, which is calculated as the ratio of the polygenic component to the residual phenotypic variance after removing the environmental component:

$$h^2_r = \frac{\sigma_g^2}{\sigma_T^2 - \sigma_E^2}.$$

Since both of these measures of heritability are used as indicators of the cumulative amount of variance explained by *all* additive genetic loci, they are assessed only for models lacking the QTL component (i.e., under the constraint $\sigma_m^2 = 0$). The residual error component, σ_e^2, includes all variance not modeled by the other components, including non-additive genetic variance, unmeasured environmental variances, and other sources of error variance.

The aforementioned components of phenotypic variance are generated by modeling the phenotype as follows:

$$y_i = \mu + \sum_{i=1}^{n} \beta_j X_{ij} + g_i + m_i + e_i,$$

where μ is the overall mean of the trait, X_{ij} is the jth covariate for the ith individual, β_j is the corresponding regression coefficient, g_i is the additive genetic effect, m_i is the effect of an arbitrary QTL, and e_i is the residual error effect. Pedigree-based maximum likelihood methods are used to estimate these parameters, and their significance is assessed via the likelihood ratio test, which compares the full model (i.e., containing the parameter to be tested) to the nested model (i.e., constraining the parameter to be tested). The test statistic asymptotically follows the chi-squared distribution with 1 degree of freedom for testing covariates (H_0: $\beta_j = 0$), and follows

a 50:50 mixture of chi-squared distribution with 1 degree of freedom and a point mass at zero for testing heritability (H_0: $h^2 = 0$) and linkage (H_0: $\sigma_m^2 = 0$). The proportion of total variance attributable to covariate effects is assessed by comparing the estimated variance in the model excluding all covariate effects to that of the model including them.

1.7.5 The LOD score

Evidence of linkage, modeled by the QTL component, σ_m^2, is assessed at 1 cM intervals across the genome. By tradition, the statistical significance of σ_m^2 is reported as a LOD score ($\log_{10}$ of the likelihood ratio), and according to guidelines set by Lander and Kruglyak (99), the LOD thresholds for determining suggestive and significant evidence of genome-wide linkage are LOD = 1.9 and 3.3, respectively. In this dissertation, we have used LOD > 2.0 to indicate suggestive linkage. Due to the limited number of recombination events occurring in the sample pedigrees, a putative QTL cannot be mapped to an exact chromosomal position, and need not occur directly at the maximum LOD score. Instead, a QTL can be mapped to a general chromosomal region. In this dissertation we have explored the one-LOD unit support interval (which is the region surrounding a linkage peak with boundaries occurring at the chromosomal positions exhibiting evidence of linkage equal to 1 unit less than the maximum LOD score) for positional candidate genes. The one-LOD unit support interval, originally recommended by Conneally et al. (103) is used in this dissertation as a rule-of-thumb for defining a linkage region, rather than a statistically rigorous confidence interval.

The variance components methods used to assess linkage assume multivariate normality of the phenotype, and deviations of the phenotype distribution from multivariate normality have

been shown to cause excessive Type I error (104). Therefore empirical LOD score adjustment based on 10,000 simulations of a fully-informative, unlinked marker was used to estimate a linear LOD score adjustment factor to guard against inflated LOD scores. Simulations have shown such adjustment adequately yields robust LOD scores (104).

1.7.6 Testing unequal genetic variances between subsets

One of the major hypotheses explored in this dissertation is whether the roles of genes on bone phenotypes of interest may differ between two groups, for example, between men and women, or between younger and older individuals. We have used an extension of the general variance components framework described above that allows genetic variances (i.e. polygenic components, σ_g^2) to differ between two groups when both are analyzed simultaneously. Details for this method have been described previously for modeling separate genetic variance in men and women (25) and pre- and postmenopausal women (24). In brief, the expected additive genetic covariance between two relatives, *i* and *j*, from different groups, A and B, is defined as:

$$\mathrm{COV}(A_i,B_j) = 2\Phi\rho_g \times \sigma_{gA} \times \sigma_{gB},$$

where Φ is the kinship coefficient between the two relatives, ρ_g is the additive genetic correlation between groups A and B, σ_{gA} is the genetic standard deviation in group A, and σ_{gB} is the additive genetic standard deviation in group B. The genetic correlation quantifies the degree to which the additive genetic variation among members of group A correlates with the additive genetic

variation among members of group B. These additional parameters allow the phenotypic variance to be partitioned into group-specific heritable components, σ^2_{gA} and σ^2_{gB}, along with group-specific residual error terms, σ^2_{eA} and σ^2_{eB}. The environmental component, σ^2_E, is modeled exactly as in the general variance components framework detailed above. Covariates are assumed to have common marginal effects on both groups; group-specific covariate effects are modeled as interactions with a dummy variable indicating individuals' membership in a group.

This expanded model was used to test two specific hypotheses regarding the role of genes in the two groups: (i) whether the group-specific additive genetic variances are equal (H_0: $\sigma^2_{eA} = \sigma^2_{eB}$), and (ii) whether the additive genetic correlation between groups was different than one (H_0: $\rho_g = 1$). Unequal additive genetic variances would imply that genes account for more phenotypic variation in one group than the other. Inter-group genetic correlation significantly less than 1 would imply that partially different suites of genes are contributing to BMD variation in groups A and B. A genetic correlation equal to zero would imply that different genes influence the trait in each group. Pedigree-based maximum likelihood methods were used to estimate model parameters. The likelihood ratio test was used to test for unequal additive genetic variances by comparing the full model (where parameters σ^2_{eA}, σ^2_{eB}, and ρ_g are estimated) to a constrained model (where $\sigma^2_{eA} = \sigma^2_{eB}$), which asymptotically follows the chi-square distribution with 1 degree of freedom. The likelihood ratio test was also used to test for genetic correlation less than 1 by comparing the full model to a constrained mode (where $\rho_g = 1$), which asymptotically follows a 50:50 mixture of a point mass at zero and a chi-squared distribution with 1 degree of freedom.

This modeling framework was utilized in Chapter 2 to test for unequal genetic variances in bone loss phenotypes between individuals younger and older than 45 years. In Chapter 5 this methodology was used to test for sex-specific additive genetic effects on cross-sectional BMD.

1.8 QUESTIONS WE EXPLORED

In this dissertation we investigated the longitudinal aspects of SAFOS to better understand the risk factors for osteoporosis. Research was conducted under the risk model (Figure 1.2) that bone strength and risk of osteoporosis-related heath outcomes are determined by the genes and environmental factors influencing peak BMD attainment in youth, and by the genes and environmental factors affecting subsequent loss with age. A number of questions regarding the roles of such genetic and environmental determinants of osteoporotic risk were explored in this project.

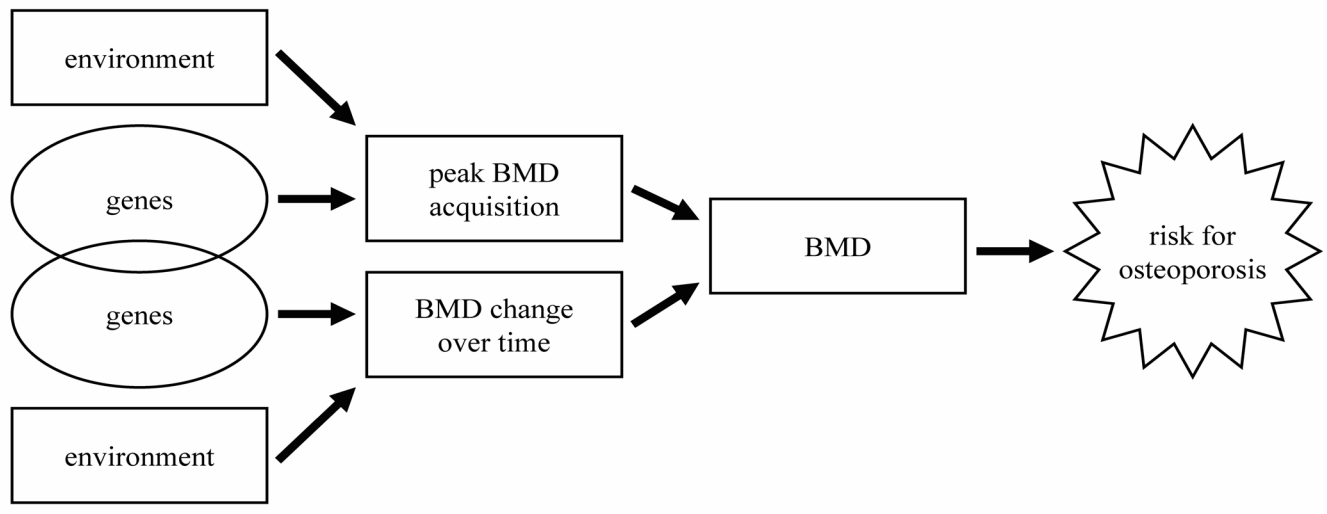

Figure 1.2. Risk model for osteoporosis

In Chapter 2 we introduce change in BMD as a quantitative trait, and ask how the rate of BMD change differs over the adult lifespan in Mexican Americans: At what age do involutive changes in BMD occur? Is the trajectory of BMD over time similar across skeletal sites and between men and women? Could distinct genes be acting on rates of BMD change at different ages?

Next, we investigated the roles of genetic and environmental factors on longitudinal change in BMD: What measured covariates have significant effects on rate of BMD change? Is rate of BMD change heritable? What specific chromosomal regions influence rate of change? Chapters 3 and 4 (formatted for journal publication) explore these questions in older (>45 years) and younger (<45 years) sub-samples of the SAFOS population.

We also examined cross-sectional measures of areal BMD at baseline and follow-up to examine the roles of environmental and genetic factors on peak BMD. Specifically, we asked: Do analyses of baseline and follow-up data show similar heritability and effects of covariates for hip and spine BMD, both overall and within the sexes? Are chromosomal regions influencing peak BMD observed at both time points? How does the concordance or discordance of results between baseline and follow-up affect interpretation of our findings? These questions are examined in Chapters 5 and 6 (formatted for journal publication).

This dissertation concludes with a discussion of our major findings and their implications for osteoporosis. In particular, we pull together results from the preceding chapters to address the questions of whether chromosomal regions influencing peak BMD overlap with those affecting bone loss and whether genes controlling early bone loss are distinct from those affecting late bone loss. These questions, along with future directions for the study of osteoporotic risk and the public health implications of this work are addressed in Chapter 7.

2.0 AGE-RELATED CHANGES IN BONE METABOLISM

2.1 INTRODUCTION

Skeletal growth occurs jointly with linear bone mineral density (BMD) accrual during youth. By age 20, adult skeleton size is usually achieved, followed by a period of bone consolidation lasting up to 15 years where BMD accretion continues, but skeletal size remains static (105). The exact timing of peak BMD acquisition is unknown and likely differs between skeletal sites, individuals, sexes, and populations (106). During the period leading up to peak attainment, change in BMD results from two processes: bone modeling (i.e. where bones are shaped through independent processes of bone deposition and removal) and bone remodeling (a.k.a. bone turnover, where bone resorption and formation are tightly coupled in a cycle of bone renewal) (107). Most cross-sectional studies indicate that peak BMD is achieved sometime during young adulthood followed by a prolonged plateau phase where BMD is maintained with little or no change (106). Other studies show varying degrees of bone loss occurring immediately after peak attainment, particularly in trabecular bone (17,92). Unlike changes leading up to peak BMD, most age-related changes in BMD after peak attainment are the result of imbalanced bone turnover, where more bone is formed than is resorbed (leading to increased BMD) or more bone resorbed than is formed (leading to bone loss) (107). Over time, bone remodeling also alters the geometry of bones, including their size, which in turn affects measurement of BMD (107).

Nevertheless, change in BMD in adults is largely an outcome of bone turnover, thus measuring change in BMD serves as indicator of bone metabolism.

Age-related bone loss occurs at all skeletal sites; however, like peak BMD acquisition, the timing of onset of bone loss is not entirely clear (108,109), and certainly differs by skeletal site, sex, and population, and may be delayed or hastened by environmental factors. During menopause, changes in hormone production result in increased bone resorption and concurrent decline in BMD, especially in trabecular bone (110). Rapid bone loss occurring in late stages of life has been documented in numerous studies (111).

Over the course of the lifespan, the relationship of BMD with age exhibits (at least) three distinct phases: initial increase in BMD in adolescents leading to peak BMD in young adults, stabilization of BMD at maturity, and decline in BMD in postmenopausal women and the elderly (109,112). Because measurable BMD in older individuals is the net result of all three of these phases, we hypothesize that osteoporosis later in life is due in part to the processes that affect BMD across the entire lifespan.

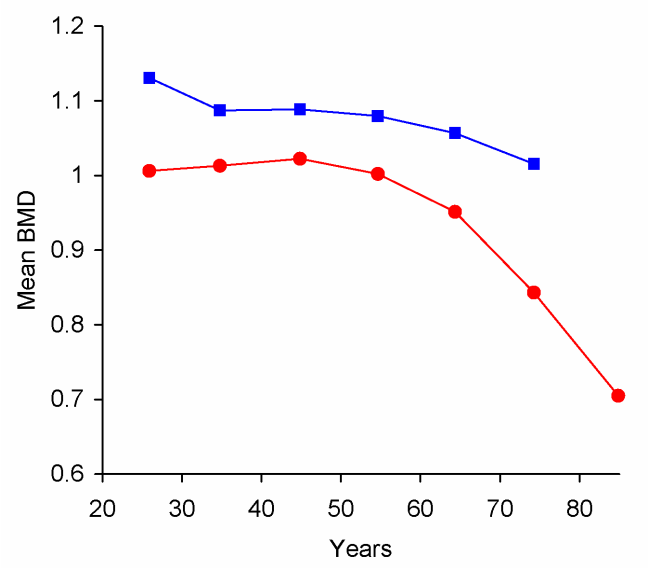

Figure 2.1. Mean total hip BMD (g/cm^2, binned by decade) by age in SAFOS men (blue squares, blue line) and women (red circles, red line)

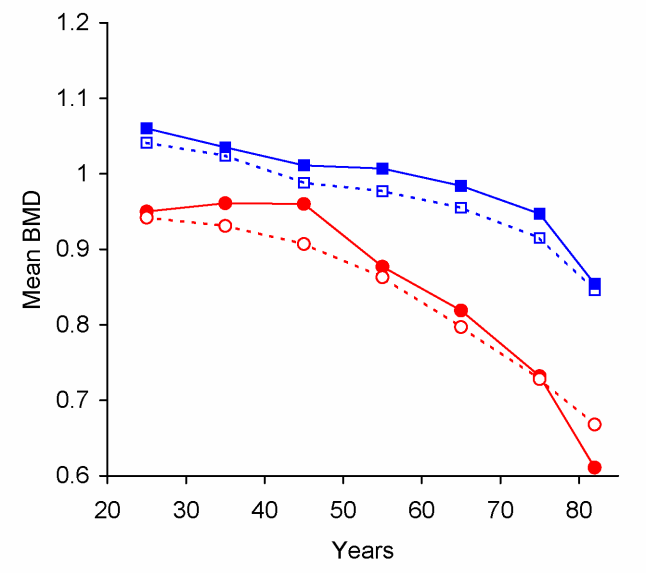

Figure 2.2. Mean total hip BMD (g/cm^2, binned by decade) by age in Mexican American men (blue solid squares, blue solid line) and women (red solid circles, red solid line) and Caucasian men (blue open squares, blue dashed line) and women (red open circles, red dashed line) in NHANES III (18)

2.2 INVOLUTIVE BONE CHANGE OCCURRING IN MIDLIFE

The apparent trajectory (based on cross-sectional data) of mean total hip BMD with age (binned by decade) in the San Antonio Family Osteoporosis Study (SAFOS) sample is shown in Figure 2.1. For comparison, the apparent trajectory (based on cross-sectional data) of mean total hip BMD in Caucasian and Mexican American participants of the National Health And Nutrition Examination Survey (NHANES III) is shown in Figure 2.2 (18). Looking cross-sectionally, the apparent trend in BMD over time shifts sometime during the fifth decade (at approximately 45 years of age), in men and women of SAFOS and NHANES.

The age at which involutive bone changes occur (i.e. the inflection point of BMD) was investigated in an extensive study by Malkin et al., (109) who collected BMD measurements in 4945 adult subjects (2430 men and 2515 women aged 18 to 100 years) from 5 ethno-geographical populations. The sex-specific age dependence of BMD was assessed by fitting many models (i.e. BMD as a mathematical function of age) using maximum likelihood methods. Models investigated included polynomial (zeroth through 4th power), exponential, logarithmic, and logistic functions, as well as piecewise combinations of constant and linear functions (i.e. two different functions applied to BMD data of participants younger and older than some age parameter). Akaike Information Criterion and likelihood ratio tests of nested models were used to determine the most parsimonious model and assess the significance of parameters. Piecewise combination models were best for women in all 5 ethno-geographic groups and for men in 4 of the 5 ethno-geographic groups (109).

Malkin et al. found that the exact age at inflection varied by sex and population from 28 years to 48 years (Table 2.1). Statistically significant pair-wise differences in the timing of involution were observed between most ethno-geographic groups for both men and women. In

particular, Asian men and women experienced a change in BMD trend at 34 and 43 years of age, respectively, whereas Caucasian men and women underwent this change at 49 and 44, respectively. These results are consistent with observations in our SAFOS sample, especially if we consider that Mexican Americans are an admixed population with of large proportions of Caucasian and Amerindian (Asian) ancestry, and that ages of inflection in Mexican American men and women may fall somewhere between those of the parent populations. Environmental differences between populations may also affect the onset of involutive bone changes.

Table 2.1. Age of involutive BMD changes as modeled by Malkin et al. in five ethno-geographic populations (109)

	Age at involution (years)	
Population	Men	Women
Slavic	34	40
Asian	34	43
Turkmenian	-	38
Caucasian	49	44
Chuvasha	28	48

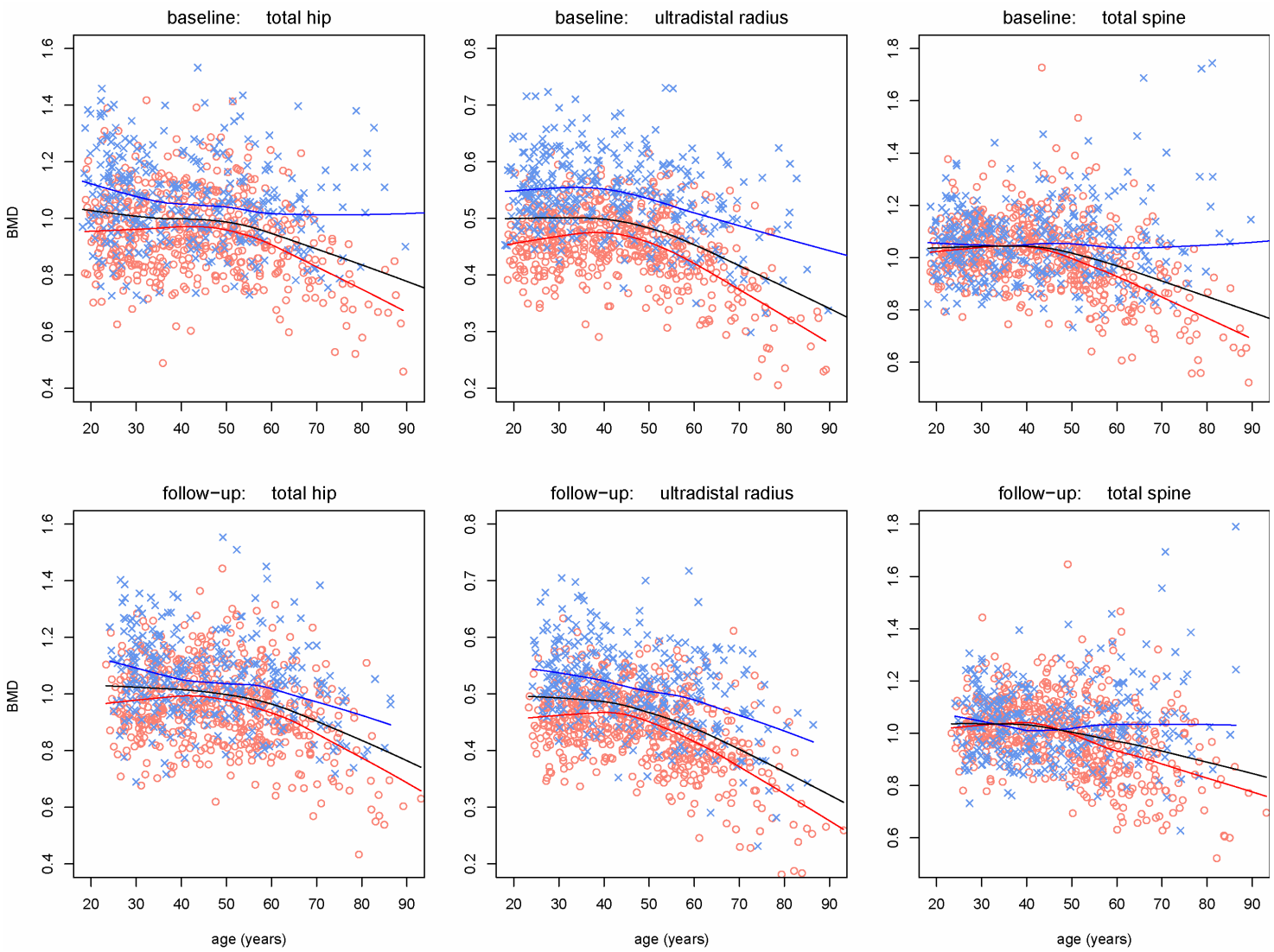

Figure 2.3. Baseline and follow-up BMD (g/cm^2) by age for total hip, ultradistal radius, and total spine in men (blue Xs) and women (red circles). Local regression (LOWESS) curves are shown for the total sample (black), men (blue), and women (red).

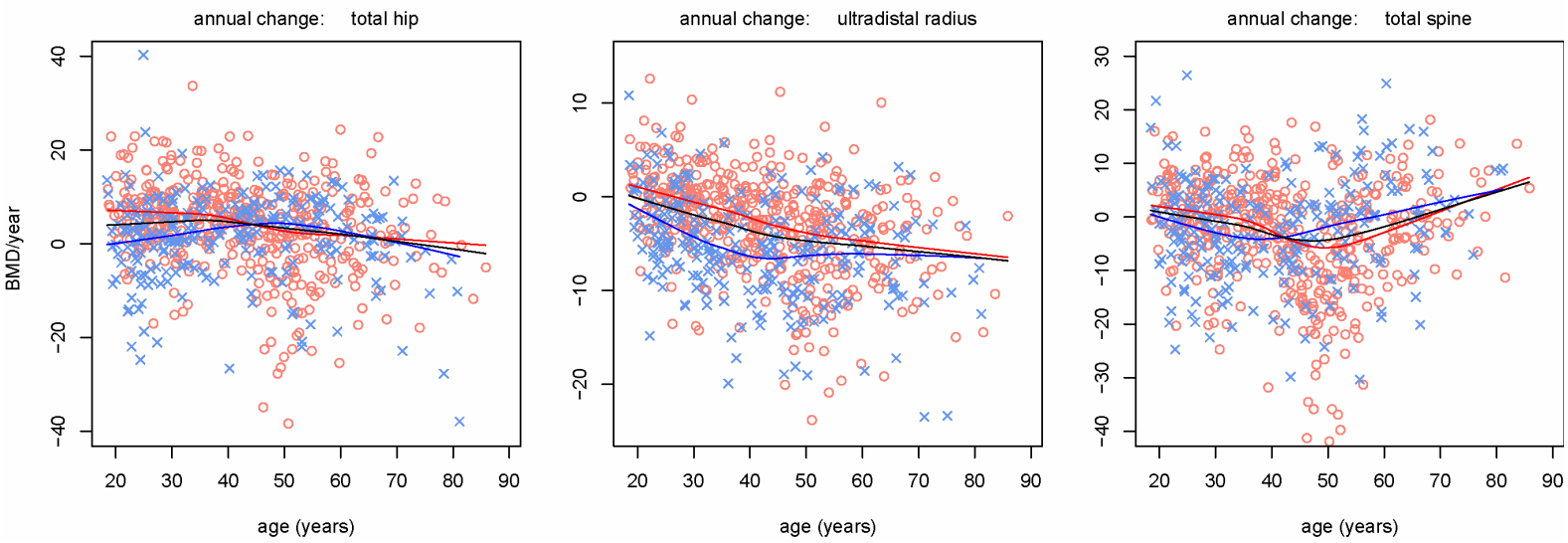

Figure 2.4. Annual change in BMD (mg/cm^2/year) by baseline age for total hip, ultradistal radius, and total spine in men (blue Xs) and women (red circles). Local regression (LOWESS) curves are shown for the total sample (black), men (blue), and women (red).

Figure 2.3 plots unadjusted BMD data for SAFOS baseline and follow-up samples and fits local regression (LOWESS) curves to represent the trend with age. Strictly speaking, the search of model-space as employed by Malkin et al. is not appropriate for our family-based SAFOS sample. However, the shift in trend of BMD over time (occurring between age 40 and 50 years) is apparent. Age of involution for baseline BMD measurements occurs at approximately 45 years for total hip, 43 years for ultradistal radius, and 45 years for total spine. Involution occurred at similar ages for follow-up BMD measurements . Note that the trend of spine BMD with age in older men appears inconsistent with bone loss. This phenomenon is possibly an artifact of inflated densitometry measurements of spine in men due to the high prevalence of osteoarthritis (resulting in growth of osteophytes, i.e. boney spurs) and aortic calcification in this age group (113-115). Figure 2.4 plots the annual change in BMD between baseline and follow-up measurements by age, with trends indicated by LOWESS regression curves. Looking longitudinally, again, the shift in trend of rates of BMD change over time is apparent between the fifth and sixth decades (at approximately 45 years of age). These observations are consistent with recent work exploring the timing of onset of loss in trabecular and cortical bone: cortical bone, in particular, undergoes a characteristic involutive shift midlife (17,92).

2.3 GENETICS ANALYSIS OF BMD ACROSS AGE RANGES

Genetic analyses of BMD, such as heritability and linkage studies, have generally focused on peak BMD, and when necessary have made linear and/or quadratic adjustment for age to model peak BMD. One problem with this approach is that it assumes a universal trajectory of BMD

over the lifespan and may lead to a loss of important data with respect to inter-individual variation in trajectory (109). In other words, such age-adjustment assumes that the rates of change in BMD over time are identical for all individuals; however, recent work has suggested that variation in rates of change in BMD are under genetic regulation (94,95,116). Therefore, this approach is not ideal.

The problem of analyzing data across a wide range of ages is even greater if the phenotype of interest is rate of BMD change over time. In this situation there is no reference point analogous to cross-sectional peak BMD, so the interpretation of change in BMD depends largely on the age range of the sample. In fact, analysis of BMD change data for samples that span multiple phases of BMD progression (i.e. peak acquisition in youth, stabilization at maturity, and decline with advanced age) is problematic, and interpretation uncertain.

Because bone metabolism changes with age, it is likely that different genes may influence changes in BMD across the lifespan. Indeed, activation, deactivation, and changes in the regulatory roles of genes may be responsible for some of the changes in bone metabolism across the lifespan. Moreover, there is no evidence that change in BMD at one point in life is predictive of change decades later. Thus, change in BMD as a measure of bone remodeling may constitute different "phenotypes" at the metabolic level during different phases of BMD progression. Therefore, the question of how to appropriately analyze family data in order to detect genetic effects, which may differ across individuals of different ages, becomes imperative to the success of a study. One option is to jointly model the genetic effects on multiple aspects of phenotype trajectory over time (e.g. separate parameters for trends before and after some age parameter). Results of a segregation analysis of cortical index (a measure of cortical thickness, which is related to BMD and shows similar age-dependency), by Karasik et al. indicated that (i)

baseline level, (ii) age at onset of involutive bone changes, and (iii) rate of decrease with age, are all under joint genetic regulation (108). One problem with this model is that it assumed all three parameters describing cortical index (i to iii) were under pleiotropic control by the same genetic factor. While this may be the case for cortical index, it may not be true for other phenotypes; instead, it is reasonable to consider that different genes may be involved in these three model parameters.

Table 2.2. Tests for difference in genetic variance between participants <45 and >45 years of age

	genetic SD (mg/cm^2/year)		
	<45 years	>45 years	p-value
total hip	2.7	8.6	0.02
ultradistal radius	3.1	5.4	0.04
total spine	2.8	5.4	0.49

Another option to reduce age-related genetic heterogeneity is to limit analysis to age ranges representing specific phases of bone metabolism. For example, separate analyses may be carried out for sub-samples younger and older than the age of onset of involutive bone changes. We tested whether this approach was sensible in the SAFOS population by modeling genetic variance (i.e. the variance due to additive genetic factors) with separate parameters for participants younger and older than 45 years. Pedigree-based maximum likelihood methods within a variance components framework were used to estimate parameters of age-specific genetic variance, $\sigma^2_{G(<45yr)}$ and $\sigma^2_{G(>45yr)}$. Details of this method have been described elsewhere (24,25). Statistical significance of the difference in genetic variance between subsets <45 and >45 years was assessed via the likelihood ratio test comparing the model with unequal genetic

variances to the model with equal genetic variances: $\sigma^2_{G(<45yr)} = \sigma^2_{G(>45yr)}$. This test statistic follows a chi-squared distribution with 1 degree of freedom. Modeling was performed using the Sequential Oligogenetic Linkage Analysis Routines (SOLAR) software (101). Significant differences in genetic variance for participants <45 and >45 years of age were observed for the total hip and ultradistal radius, but not the total spine (Table 2.2).

2.4 CONCLUSIONS

Several lines of evidence indicate that bone metabolism changes over time, with at least three distinct phases (i.e. acquisition, stabilization, and age-related decline) occurring during the adolescent and adult lifespan. However questions remain regarding when these changes occur, and the reasons for them. We hypothesize that genetic factors may be involved in different capacities during different phases of bone metabolism. This assertion is supported by statistical modeling of the age-dependency of BMD showing that distinct trends over time occur during different stages of life (109). Such trends are apparent whether looking at cross-sectional or longitudinal BMD data in the SAFOS population. Moreover, recent work indicates that the metabolism of the two types of bone, trabecular and cortical, show independent changes over time (17,92). Lastly, genetic modeling of SAFOS participants <45 and >45 years reveals statistically significant differences in the magnitude of additive genetic variance for younger and older cohorts.

Altogether, these observations suggest that genetic heterogeneity with regard to BMD, and in particular, the rate of change in BMD over time, may exist between phases of bone metabolism. Our hypothesis, which is that variation in BMD and rate of BMD change over time

is due, in part, to the effects of separate genes acting on bone metabolism in younger and older individuals, was explored by performing separate analysis in individuals <45 and >45 years of age. Results from these analyses, detailed in Chapters 3 to 6, support this hypothesis for change in BMD. While questions still remain, we believe the SAFOS study has made important contribution to our understanding of the genetic, as well as environmental, determinants of BMD and bone loss.

3.0 HERITABILITY AND LINKAGE ANALYSIS FOR CHANGE IN BONE MINERAL DENSITY IN OLDER MEXICAN AMERICANS

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3.1 ABSTRACT

The genetic contribution to age-related bone loss is not well understood. We estimated that genes accounted for 25-45% of variation in 5-year change in bone mineral density in men and women. An autosome-wide linkage scan yielded no significant evidence for chromosomal regions implicated in bone loss.

Introduction: The contribution of genetics to acquisition of peak bone mass is well documented, but little is know about the influence of genes on subsequent bone loss with age. We therefore measured 5-year change in bone mineral density (BMD) in 300 Mexican Americans (>45 years of age) from the San Antonio Family Osteoporosis Study to identify genetic factors influencing bone loss.

Methods: Annualized change in BMD was calculated from DXA measurements taken 5.5 years apart. Heritability of BMD change was estimated using variance components methods and autosome-wide linkage analysis was carried out using 460 microsatellite markers at a mean 7.6 cM interval density.

Results: Rate of BMD change was heritable at the forearm (h^2_r = 0.31, p = 0.021), hip (h^2_r = 0.44, p = 0.017), spine (h^2_r = 0.42, p = 0.005), but not whole body (h^2_r = 0.18, p = 0.123). Covariates associated with rapid bone loss (advanced age, baseline BMD, female sex, low baseline weight, postmenopausal status, and interim weight loss) accounted for 10% to 28% of trait variation. No significant evidence of linkage was observed at any skeletal site.

Conclusions: This is one of the first studies to report significant heritability of BMD change for weight-bearing and non-weight-bearing bones in an unselected population and the first linkage scan for change in BMD.

3.2 INTRODUCTION

Osteoporosis is a skeletal condition of major public health significance, contributing toward the risk of fragility fracture in women and men of all populations. The degenerative disorder and associated fragility fractures have devastating effects on health, resulting in substantial morbidity, and increased mortality for hip and vertebral fractures (1). Bone mineral density (BMD), as the foremost determinant of bone strength and major predictor of future fractures, has been extensively studied to help identify the environmental (3,29,117) and genetic factors (27,28,30,42) influencing risk for osteoporosis.

While the contribution of genetics to variation in BMD is widely acknowledged, the mechanisms by which genetic factors affect BMD are not well understood. Bone is dynamically maintained through the cycle of bone formation and resorption, with changes in bone mass resulting from an imbalance of bone turnover processes. In general, bone turnover yields a net increase in BMD during adolescence and young adulthood leading to peak bone mass attainment, followed by a net decrease in BMD resulting in the subsequent loss of bone with advanced age. Cross-sectional study designs cannot sufficiently distinguish between processes leading to peak BMD acquisition versus loss with age, and this has been a persistent limitation of cross-sectional epidemiological and genetic studies of BMD, particularly those carried out in older individuals since such studies cannot allow for variation in rates of change in BMD during aging.

Longitudinal studies have shown that weight, interim change in weight, alcohol consumption, smoking, sex, estrogen replacement therapy, menopausal status, exercise, calcium intake, and serum vitamin D level may affect change in BMD over time, and that rates of BMD change may differ among skeletal sites (85-88,118). Rate of bone loss as a risk factor for fracture independent of bone mass has recently been reported in a cohort of postmenopausal women (mean age of 62 years) (119), reinforcing the clinical importance of change in BMD for bone health.

The degree to which genes affect the rate of BMD change over time remains largely unresolved (32). To date, very few studies have explicitly addressed the role of genetics for change in bone traits over time. One investigation of 25 monozygotic (MZ) and 21 dizygotic (DZ) pairs of twins reported no evidence of heritability for decline in radial bone mass over 16 years (26), while a second study reported greater similarity between 21 MZ than 19 DZ twin pairs for annualized percent change in lumbar spine and Ward's triangle over 1 to 5 years (93). A larger study of 177 monozygotic and 185 dizygotic female twin pairs (ages 45 to 82), revealed evidence for genetic effects on 5-year change in BMD at the lumbar spine, whole body, and forearm, but not hip (95). Finally, a study of premenopausal sisters from 178 sibships demonstrated significant heritability (h^2 = 0.29 to 0.35) of 6-year change in femoral neck bone mineral content (BMC) and BMD (94). Although there is some consistency among the results of these studies, there are also differences; for example, two studies report significant genetic effects on change in hip BMD (93,94), and one does not (95). These differences may be due to the small sample sizes of some studies, length of time elapsed, age range of subjects, and study design (twins versus siblings). Thus, additional research is needed because of the inconsistency of results regarding the heritability of BMD change at all high-risk fracture sites (i.e., spine, hip,

and forearm) in postmenopausal women. Furthermore no studies have clearly demonstrated heritability of BMD change in men of any age, or performed linkage analysis to find QTLs influencing BMD change in any population.

The San Antonio Family Osteoporosis Study (SAFOS) was designed to investigate the influence of genes and environmental factors on BMD and change in BMD over time in Mexican Americans. In the current report, we assessed the cumulative effects of genes (i.e. additive heritability) and performed autosome-wide linkage analyses on 5-year longitudinal change in BMD (ΔBMD) at several skeletal sites among 300 men and women (>45 years of age) in 32 extended pedigrees.

3.3 METHODS

3.3.1 Subjects and Data Collection

Recruitment and data collection for the baseline phase of the SAFOS has been previously described in detail (30). The families included for study were selected without regard for health outcomes and represent a relatively random sample of low income families from urban San Antonio. In brief, 34 probands of Mexican American descent aged 40 to 60 were identified, and all first, second, and third degree relatives of probands and their respective spouses were invited to participate in the study. The only criterion for inclusion of probands was that they have large extended families (>6 members) in the San Antonio area. Anthropometric, medical, and body composition data were collected during medical examinations at baseline between 1997 and 2000. Body composition data and select covariates were reassessed during a follow-up

examination (2002-2006) occurring 3 to 8 years later (mean = 5.5 years). Lifestyle, medical history, and reproductive history data were collected via questionnaire. Of the original sample of 895 individuals from 34 families considered in this study (ages 18 to 98), 724 (80.9%) have currently been re-enrolled for follow-up.

The present analyses were confined to the subsample of individuals aged >45 years at baseline (n = 370 at baseline, n = 300 at follow-up). This cohort comprises age-eligible members of 32 families, among whom are 173 sibling pairs, 126 first-cousin pairs, and 189 other relative pairs. Two of the initial 34 families did not include any relative pairs in this age range and were thus excluded from these analyses. The heritability and linkage analyses carried out in this study exclude individuals younger than 45 years at baseline because differences in the biological processes that influence bone turnover between younger and older individuals may be due, in part, to different genes contributing to bone turnover in younger and older individuals. This speculation is supported by (unpublished) genetic modeling in our sample, showing unequal genetic variances between individuals younger and older than 45 years for change in forearm ($p = 0.04$) and hip BMD ($p = 0.02$).

Measurements of BMD of the total hip, total lumbar spine (L1-L4), ultradistal radius, 33% ulna (measured at one-third its total length from the distal end), and whole body were obtained by dual-energy x-ray absorptiometry (DXA) at both baseline and follow-up. Ultradistal radius and 33% ulna sites were analyzed separately because these sites are composed of differing proportions of cortical and trabecular bone: 33% ulna, like total hip, is largely comprised of cortical bone, whereas ultradistal radius, like total spine, is primarily trabecular bone. During the interim between baseline and follow-up clinic visits, DXA equipment was upgraded from a pencil beam Hologic 1500W to a fan beam Hologic 4500W absoptiometer (Hologic, Inc.,

Bedford, MA). A software upgrade was also included for compatibility of scoring algorithms between the two machines. Cross-calibration of absorptiometry equipment used at baseline and follow-up indicated excellent agreement between measurements taken from Hologic 1500W and 4500W scanners (e.g., $R^2 > 0.99$ for measurements taken at the hip and spine from the two scanners on the same 10 subjects) and thus the effect of this equipment change on statistical analyses and results should be minimal (see 3.6 Supplemental Material).

For quality control, the same technician operated all equipment, and phantom measurements were taken daily to guard against measurement drift. Patient positioning was performed according to the Hologic positioning protocol; baseline and follow-up scans were all compared by the same reviewer, and when necessary, scans were re-analyzed to prevent non-overlapping regions of interest.

Covariates considered in this study include sex, age, age^2, age × sex, age^2 × sex, site-specific baseline BMD, baseline weight, annualized change in weight, annualized change in height, and baseline and follow-up measures of menopausal status (defined by surgical menopause or 1 or more elapsed years without menstrual period). Measurements of all baseline covariates were previously described in Mitchell et al. (30); follow-up measurements of covariates were assessed identically to the baseline measures. Annualized change in BMD (ΔBMD), height, and weight was calculated as the difference between follow-up and baseline measurements divided by the exact elapsed time between clinic visits.

3.3.2 Genotyping

Genotyping for SAFOS was carried out as previously described in detail (42) for the San Antonio Family Heart Study (SAFHS, the parent project to SAFOS) January 2007 genetic map

build: 460 highly polymorphic microsatellite markers across all chromosomes were genotyped, and genetic maps were assembled via the program CRI-MAP (120) and confirmed using marker locations specified by deCODE (deCODE genetics, Reykjavik, Iceland). Mean inter-marker distance was 7.6 cM, ranging from <0.1 cM to 15.7 cM (Haldane).

3.3.3 Statistical analyses

The two goals for analyses presented herein were (i) to determine the extent to which genetic and measured environmental factors contribute to the phenotypic variation in ΔBMD at different skeletal sites, and (ii) to perform an autosome-wide linkage scan for regions affecting ΔBMD. Prior to analyses, the distributions of ΔBMD phenotypes and covariates were assessed, and data points greater than 4 standard deviations from trait and covariate means were excluded (0 to 3 observations removed per trait or covariate).

As previously described in detail (24,30), heritability of ΔBMD was estimated using variance decomposition methods, which model phenotypic variation in ΔBMD at each bone site as a function of effects attributable to the measured covariates, additive genetic (based on expected allele sharing between pairs of relatives), and unmeasured error components. This model takes the general form $y_i = \mu + \sum_{i=1}^{n} \beta_j X_{ij} + g_i + e_i$, where y_i is the annualized ΔBMD for the ith individual, μ is the sample mean ΔBMD, X_{ij} is the jth covariate for the ith individual, β_j is the corresponding regression coefficient, g_i is the additive genetic effect, and e_i is the residual error effect. Pedigree-based maximum likelihood methods were used to estimate these parameters, from which residual narrow-sense heritability (h^2_r, the proportion of total trait variance due to the additive genetic component after adjusting for environmental covariates) was

determined. The likelihood ratio test was used to assess the significance of model parameters by comparing the full model (all covariates and additive genetic effects) with a nested model lacking a specific component. The test statistic asymptotically follows the chi-squared distribution with one degree of freedom for testing covariates, and follows a 50:50 mixture of chi-squared distribution with one degree of freedom and a point mass at zero for testing heritability. Power was 94%, 84%, and 66% to detect true heritability of 0.45, 0.35, and 0.25, respectively at $\alpha = 0.05$. The analysis of each ΔBMD phenotype was limited to individuals with observed data for all retained covariates. The proportion of total variance attributable to covariates was estimated by comparing the estimated variance in the model excluding all significant covariates to that of the model including significant covariates.

Multipoint linkage scans were performed using the variance components method, which extends the above model by also including the effect of a presumed QTL, σ_m^2, as a component of ΔBMD genetic variance. Maximum likelihood methods were used to estimate σ_m^2 based on the expected covariance of relatives due to their identity-by-descent (IBD) at a given marker (two-point analyses) or at an arbitrary chromosomal location (multipoint analyses) in tight linkage with the presumed QTL. A Markov Chain Monte Carlo algorithm, as implemented in Loki, was used to calculate multipoint IBD probabilities using data from all genotyped individuals (102). The likelihood ratio test was used to compare the linkage model to the polygenic (i.e. no linkage, $\sigma_m^2 = 0$) model, and findings were reported in LOD scores (i.e. $\log_{10}$ of the likelihood ratio). To remedy the potential consequence of phenotype distribution on calculated LOD scores, 10,000 simulations of an unlinked marker were performed and linkage analyses carried out on each to determine the empirical LOD score distribution for each phenotype. Linear LOD score adjustments according to the empirical distribution were then applied to our findings (104).

Power to detect linkage was low: 20% and 33%, respectively, to detect a QTL describing 35% of phenotype variance at a LOD threshold of 2.0 and 1.5. Genetic analyses were performed using the Sequential Oligogenetic Linkage Analysis Routines (SOLAR) software (101). For illustrative purposes, histograms and LOD score plots were created in R (R Foundation for Statistical Computing, Vienna, Austria).

3.4 RESULTS

Population characteristics of the 300 individuals in our study are summarized in Table 3.1. Mean length of follow-up was 5.5 years and ranged from 3.2 to 8.0 years. The prevalence of obesity in the sample was high, as indicated by mean body mass index (BMI) for men and women of 31.1 and 32.9 kg/m^2, respectively. Distributions of annualized ΔBMD for different skeletal sites, calculated from measurements taken at baseline and follow-up, are depicted in Figure 3.1. Negative values indicate average yearly decline in BMD per year, whereas positive values indicate average yearly gain. BMD declined, on average, for lumbar spine, ultradistal radius, and whole body, but not 33% ulna or total hip. The differences in magnitude and direction of ΔBMD across skeletal sites reflects the site-specific consequence of aging on bone; indeed, correlations for ΔBMD among skeletal sites are low ($r = 0.15$ to 0.41). Also, mean ΔBMD of the spine differed markedly between men and women ($p < 0.001$), possibly due to the high prevalence of osteoarthritis and aortic calcification in men, leading to inflated densitometry values with increasing age (113-115).

Table 3.1. Mean population characteristics (SD) [95% CI]

trait	total sample n = 300		women n = 197		men n = 103	
length of follow-up (years)	5.5	(0.6)	5.5	(0.6)	5.4	(0.6)
demographic						
age (years)	56.7	(8.8)	56.3	(8.7)	57.6	(9.0)
lifestyle						
alcohol consumption (%)	27.0	-	15.7	-	48.5	-
smoking history (%)	17.3	-	9.6	-	32.0	-
medical						
diabetes (%)	28.3	-	28.4	-	28.2	-
reproductive						
oral contraceptives (%)	0.7	-	1.0	-		-
post-menopausal (%)	41.2	-	62.8	-		
anthropometric						
height (cm)	160.0	(8.9)	155.2	(5.4)	169.1	(6.9)
weight (kg)	82.7	(18.8)	79.3	(17.4)	89.3	(19.7)
BMI (kg/m^2)	32.3	(6.6)	32.9	(6.9)	31.1	(6.0)
change in height (cm/year)	-0.12	(0.30)	-0.14	(0.28)	-0.07	(0.32)
change in weight (kg/year)	0.00	(1.37)	0.09	(1.19)	-0.19	(1.66)
change in BMI (kg/m^2/year)	0.05	(0.53)	0.10	(0.50)	-0.04	(0.58)
BMD (g/cm^2)						
total hip	0.96	(0.16)	0.93	(0.15)	1.02	(0.15)
total spine	1.01	(0.17)	0.97	(0.16)	1.07	(0.17)
ultradistal radius	0.47	(0.08)	0.44	(0.07)	0.52	(0.07)
33% ulna	0.67	(0.11)	0.62	(0.07)	0.77	(0.08)
whole body	1.10	(0.13)	1.05	(0.12)	1.19	(0.10)
annual BMD change (mg/cm^2/year)						
total hip	0.3	[-.09, 1.5]	-0.2	[-1.8, 1.3]	1.2	[-0.6, 3.1]
total spine	-4.6	[-6.0, -3.2]	-6.9	[-8.6, -5.2]	-0.2	[-2.3, 1.9]
ultradistal radius	-5.8	[-6.5, -5.2]	-5.2	[-6.0, -4.8]	-7.0	[-8.1, -5.9]
33% ulna	7.0	[6.2, 7.8]	6.3	[5.5, 7.2]	8.3	[7.0, 9.6]
whole body	-5.6	[-6.5, -4.6]	-4.3	[-5.6, -3.1]	-7.9	[-9.3, -6.6]

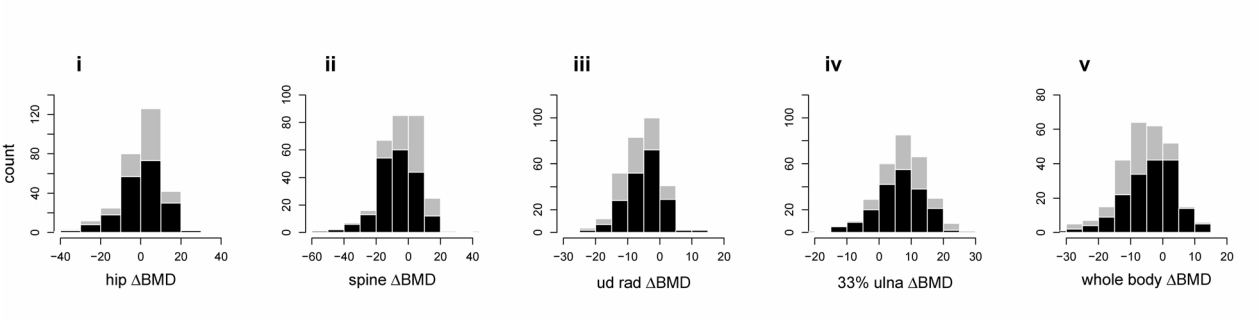

Figure 3.1. Distributions of annual change in BMD (mg/cm^2/year) for (i) total hip, (ii) total lumbar spine, (iii) ultradistal radius, (iv) 33% ulna, and (v) whole body. Gray bars represent total sample (n = 300); black bars represent the subset of women (n = 197).

Table 3.2 shows for each bone site the residual heritability of ΔBMD after adjusting for sex, age, age^2, age × sex, age^2 × sex, baseline BMD, baseline weight, menopausal status, interim change in weight, and (for spine and whole body ΔBMD only) interim change in height. In light of the well-documented bone loss following menopause, we adjusted ΔBMD for baseline post-menopausal status to correct for women having already undergone transient menopause-related bone loss, as well as interim incidence of menopause to correct for women undergoing rapid menopause-related bone loss during the years of follow-up. After incorporating covariates, we observed significant residual heritability for ΔBMD of the total hip, total spine, and 33% ulna (h^2_r = 0.31 to 0.44; $p < 0.03$ for all). Additionally, we observed modest residual heritability for ultradistal radius ΔBMD (h^2_r = 0.25, p = 0.06), but not whole body ΔBMD (h^2_r = 0.18, p = 0.12). Approximately 10% to 30% of total variation in ΔBMD was attributable to covariates. Heritability of unadjusted ΔBMD (not shown in table) was similar to heritability after covariate adjustment for all bone sites.

Table 3.2. Residual heritability of BMD change.

BMD site	n	h^2_r	SE	p-value	R^2
total hip	272	0.44	0.24	0.017	0.19
total spine	272	0.42	0.18	0.005	0.28
ultradistal radius	277	0.25	0.18	0.064	0.10
33% ulna	277	0.31	0.17	0.021	0.11
whole body	253	0.18	0.17	0.123	0.16

h^2_r = residual heritability
R^2 = proportion of variation attributable to covariates: sex, age, age^2, age × sex, age^2 × sex, baseline BMD, baseline weight, baseline post-menopausal status, interim menopause, interim change in weight, interim change in height (included only for spine and whole body **Δ**BMD)

Whole genome multipoint linkage scans were performed for ΔBMD at each skeletal site (Figure 3.2). No evidence of linkage was detected at genome-wide significance; the greatest linkage signal was observed for 33% ulna ΔBMD with a LOD score of 1.90 at 81 cM on chromosome 3p (unadjusted p = 0.0018). This region has previously been implicated in lumbar spine BMD in unselected twins and extremely discordant or concordant sib pairs (54), and for general BMD (i.e. not skeletal site-specific) in a meta-analysis of 11 whole-genome BMD scans (59). A novel signal for hip ΔBMD with a LOD score of 1.75 at 103 cM on chromosome 6q was also observed (unadjusted p = 0.0008).

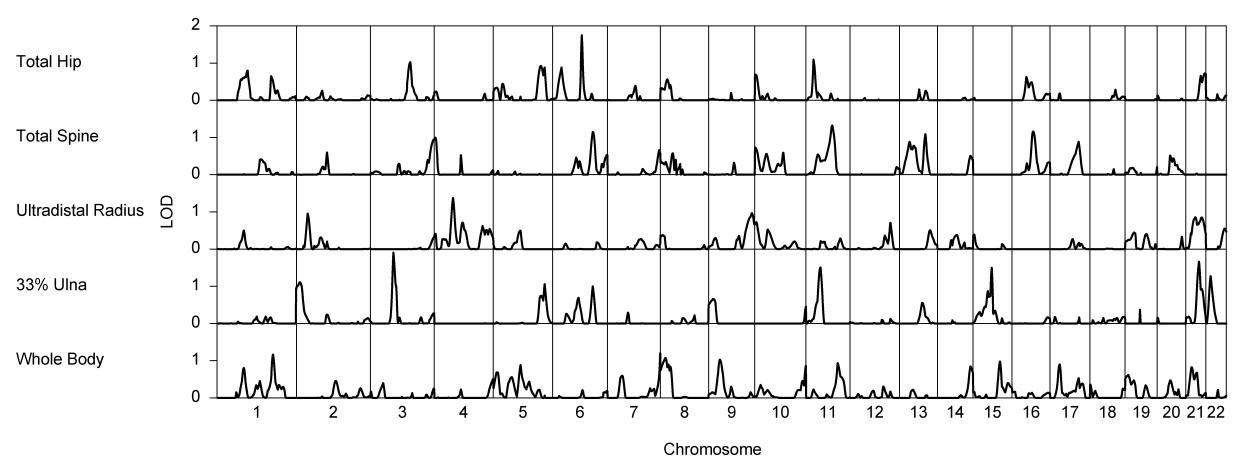

Figure 3.2. Multipoint LOD scores for change in BMD (mg/cm^2/year) across chromosomes 1 to 22.

3.5 DISCUSSION

Numerous family and sibling studies of peak BMD have been performed in a variety of populations, and these studies have universally shown the high heritability of peak BMD (17,24,25,29,30). Many whole genome linkage scans have also been performed, with QTLs reported at a number of chromosomal regions, though specific QTLs have rarely been replicated across studies (27,28,42,48,50,51,54,58,121,122), probably due, in part, to genetic heterogeneity among the populations studied. Analyses of BMD data from these previous studies, however, cannot adequately distinguish between loci affecting loss of BMD with age and those affecting the acquisition of peak bone mass occurring in young adulthood. Moreover, the models of BMD variation in these cross-sectional family studies generally assume universal rates of change with age (i.e. by adjusting BMD for age and/or age^2) thereby further reducing their ability to find genes that influence individual rates of change. In the current study, we have directly calculated ΔBMD from longitudinal measurements, to better investigate the role of genes on ΔBMD.

Our study provides support that the rate of bone loss in middle-to-older-aged Mexican Americans is heritable. Specifically, we estimated that genes account for 25% to 44% of residual variation in ΔBMD for three bone sites (hip, spine, and forearm) at high risk of fracture. The confidence intervals surrounding these estimates, however, are wide – a feature that is partly a consequence of our sample size (n = 300) of adults >45 years of age from large multigenerational families, and partly due to the measurement uncertainty for ΔBMD. We have reported heritability estimates for the combined cohort of men and women aged >45 years; heritability estimates for ΔBMD of similar magnitude were obtained when we restricted analysis to women only (n = 197), with the exception that heritability of ΔBMD at the ulna was reduced and did not differ significantly from zero (results not shown). Sample size limitations precluded performing genetic modeling of ΔBMD in men alone. In general, heritability estimates of ΔBMD in our sample (both overall, and in the women-only subset) are similar in magnitude to recently published estimates obtained in both premenopausal (94) and peri- and post-menopausal women (95), with the exception that, unlike in our study, significant genetic effects on bone loss of the hip were not observed for the latter (95).

Though significantly heritable, our genome-wide linkage scan for ΔBMD revealed no strong evidence for QTLs (including in the women-only subset; results not shown). However, power in our sample was very low to detect QTLs having relatively modest effects (e.g. at a LOD threshold of 2.0 we have 20% power to detect a locus accounting for 35% of residual phenotypic variance), and it is likely that QTLs of modest effect sizes were missed.

While the cumulative influence of genes on ΔBMD is large, the contribution of environmental factors is also important; measured covariates accounted for 10% to 28% of phenotypic variation. Associations with BMD of several of the environmental correlates

identified in this study, including age, female sex, postmenopausal status, low body weight, and weight loss, have been detected in previous studies (85,86,118,123). Interestingly, we observed differences in magnitude and direction for ΔBMD across skeletal sites, as has been observed by others, although not always for the same sites [9-11]. Unfortunately, there are insufficient reports of ΔBMD across multiple age ranges and ethnic groups to develop hypotheses regarding potential mechanisms for these differences at this time. However, this result, combined with the observed differences in heritability and variation attributable to environmental correlates among bone sites, suggests that factors regulating 5-year ΔBMD may vary across the skeleton.

A major strength of this study is the use of extended families and longitudinal measurements of BMD to investigate the genetics of bone loss. Previous linkage scans for BMD, of which there have been many, have been ill-suited to find genes affecting change, and all previous attempts to estimate the heritability of ΔBMD have been carried out exclusively in twins or siblings (26,93-95). By using information from many types of relative pairs (cousins, avuncular pairs, etc.) in addition to siblings, our estimates of heritability more accurately reflect the truly genetic factors affecting ΔBMD by reducing the contribution of familial non-genetic factors such as household effects to our heritability estimation. Other strengths of this study are the inclusion of both axial (weight-bearing) and peripheral (non-weight-bearing) skeletal sites, and the incorporation into our models of important covariates previously shown to have potential effects on ΔBMD. Furthermore, this is one of the first studies to consider ΔBMD in a population of Mexican descent and, to our knowledge is the only study, to date, reporting a linkage scan for a change in bone phenotype.

Despite these strengths, several limitations of this study must be acknowledged. Foremost is the fact that different absorptiometers were used to collect baseline and follow-up

BMD measurements. However, the BMD scoring algorithms were standardized to the degree possible and our cross-calibration experiment (albeit in a limited number of subjects) confirmed outstanding agreement in measurements between the two scanners (see 3.3 Methods and 3.6 Supplemental Material). Other limitations and sources of error for this study include the reduced sample size available for follow-up (86.5%) and the possibility of non-random loss to follow-up with respect to bone health. Also, power to detect significant linkage was limited, and our sample size and methods preclude direct detection of gene × environment interactions (in particular, gene × sex and gene × menopausal status interactions). Analyses could not be performed separately in pre- and postmenopausal women (again, due to sample size); however, the similarity of our results with those in both pre- and postmenopausal women in other studies (94,95) suggests that the impact of this limitation may be not be critical. Finally, there are inherent drawbacks to using DXA to assess BMD, notably the estimation of bone mineral content from a two-dimensional projection, which fails to precisely account for the size (depth) of bone (124). These limitations, however, would reduce our chances of detecting heritable effects or QTLs, but would not inflate them.

In conclusion, this study provides evidence that ΔBMD is heritable for several skeletal sites in middle-aged men and women. Our results corroborate recent findings for significant heritability of change in femoral neck BMD in premenopausal women (94), as well as other previous twin studies (26,93) and racial comparisons (8,14). Moreover, we report on the genetics of bone loss in Mexican Americans, a population that is under-represented in the osteoporosis literature. While evidence for the heritability of age-related ΔBMD is mounting, the localization of QTLs, and identification of specific genetic factors contributing to variation in

bone loss has yet to be realized. Such genes represent a novel area for investigation into the risk factors for osteoporosis.

3.6 SUPPLEMENTAL MATERIAL

3.6.1 DXA measurement and cross-validation

Due to an upgrade in equipment, densitometry was carried out on a pencil-beam Hologic Model 1500 at baseline examination (1997-2000) and a fan-beam Model 4500W at follow-up examination (2002-2006). For both densitometers, areal BMD was calculated by manufacturer's software as per current recommendations by dividing bone mineral content (g) by the projected area of the region scanned (cm^2). Precision of pencil-beam DXA was 0.009 g/cm^2 for spine, 0.007 g/cm^2 for total hip, and 0.002 g/cm^2 for the manufacturer's spine phantom. Precision of fan-beam DXA was 0.006 g/cm^2 for spine, 0.007 g/cm^2 for hip, and 0.002 g/cm^2 for radius. Based on the precision of our equipment, least significant change (at 95% confidence) was 3.2 mg/cm^2/year for hip, 3.1 mg/cm^2/year for spine, and 1.1 mg/cm^2/year for forearm DXA measurements.

To address the comparability in our study of measurements taken from Hologic 1500 and 4500W scanners, we performed cross-calibration of absorptiometry equipment used at baseline and follow-up on 10 participants. Measurements obtained from the two scanners showed near-perfect agreement (R^2 values = 99.95%, 99.81%, and 99.87% for spine, total hip, and femoral neck sites, respectively; $p < 10^{-13}$ for all). Moreover, regression slopes (0.99, 0.99, and 1.01, respectively, for spine, total hip, and femoral neck; $p < 10^{-13}$ for all) and paired T-tests ($p > 0.1$,

for all) revealed no evidence of systematic or mean differences between absorptiometers. This evaluation of equipment indicates that measurements from Hologic 1500 and 4500W scanners used in this study are comparable, and that 5-year change in BMD can be adequately calculated. Cross-calibration was not performed for forearm BMD, although we expect that measurements at this site are equally comparable.

3.6.2 Robustness of methods to measurement error

Though we found no evidence to suggest that systematic differences exist in the measurements between scanners, we have nonetheless employed statistical methods that are robust to potential differences. If present, such bias (e.g. systematic measurement error) would decrease our power to detect covariate effects and attenuate our estimate of heritability and linkage, but should not otherwise affect our results. That is, machine differences leading to biased estimates of BMD change could prevent us from assessing environmental and genetic factors affecting BMD change, but would not produce false positive results or lead to overestimation of effects sizes. Our findings, therefore, are conservative. Furthermore, we recognize that deviations from absolute agreement between machines would not adversely affect the genetic analyses as long as the *relative* BMD between individuals as measured by each scanner is accurate (i.e. variances are comparable). In other words, as long as BMD measured for an individual at baseline is accurate relative to the baseline measurements of the rest of the study sample, and the same holds for measurements at follow-up, the estimation of heritability will be unaffected by inter-machine differences. This is because the genetic modeling used to assess heritability decomposes the trait variance into genetic and environmental components irrespective of the trait mean.

To demonstrate this point, we removed the possibility of any inter-machine effects by first standardizing (mean = 0, SD = 1) the BMD measurements in our sample separately at baseline and follow-up, and then analyzing yearly change in standardized values. This process retains the information of individuals' measurements relative to the sample, but not of the absolute magnitude of measurements. In doing so, potential unknown machine differences that could invalidate direct inter-machine comparisons are avoided. Results (not shown) of change in standardized BMD are very similar to the absolute change reported herein. Likewise, results (not shown) of percent change in BMD were also similar.

3.6.3 Attenuation of heritability due to measurement error

The precision of DXA, which is excellent when looking at cross-sectional BMD measurements, is poor when looking at change over time, leading to considerable uncertainty of actual rates of change. Therefore, rates of change assessed in this study are notably crude, with a large percentage of observations being less than our measurement uncertainty (i.e. no measurable change). Since noise constitutes a substantial portion of the variation in observed rates of change, our results represent a considerable underestimation of the heritability of observed BMD change compared to that of true change (free of such measurement error) (94). Noise due to our crude assessment of BMD change effectively drowns the heritability signal, and diminishes our ability to detect linkage.

3.6.4 Parallel analysis of the subset aged 45 to 65 years

As previously discussed, the question of choosing the appropriate range of ages to analyze (to reduce genetic heterogeneity) is imperative to the success of this study. We presented results, above, for the cohort of men and women >45 years of age. However, analysis of a narrower age range (possibly limited to a more homogenous phase of bone metabolism with regard to genetics) may improve our ability to detect the genetic contribution to ΔBMD. To this end, we have repeated analyses while restricting our sample to individuals 45 to 65 years at baseline (n = 243). This cohort comprises members of 32 families, among which are 138 sibling pairs, 112 first-cousin pairs, and 86 other relative pairs. Individuals older than 65 years at baseline were excluded primarily based on observed differences in the epidemiology of ΔBMD between individuals 45 to 65 and >65 years. Unlike in individuals 45 to 65 years of age, ΔBMD declined rapidly with age in those older than 65 years (Figure 3.3), which suggests that individuals >65 years may have entered a different phase of bone metabolism, possibly influenced by different genes.

The cumulative effect of genetic factors on ΔBMD in those aged 45 to 65 years was assessed while simultaneously modeling the effects of the following covariates: sex, baseline BMD, baseline weight, baseline post-menopausal status, interim menopause, interim change in weight, and (for spine and whole body only) interim change in height. Heritability estimates of ΔBMD for total hip, lumbar spine, midpoint and ultradistal radius, and whole body are shown in Table 3.3.

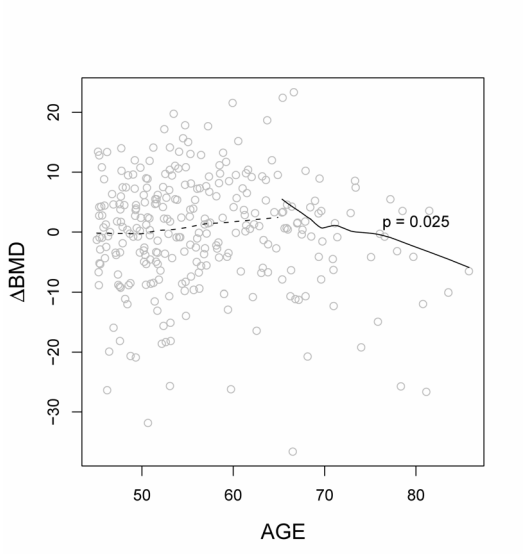

Figure 3.3. Change in total hip BMD by age. Local regression curves shown for participants 45 to 65 years of age (dotted line) and >65 years of age (solid line). Rates of change are significantly associated with age in participants >65 years of age (p = 0.025), but not in participants 45 to 65 years of age.

Figure 3.4 depicts the results of genome-wide linkage scans for ΔBMD at each skeletal site. A suggestive QTL (maximum LOD = 2.5 at 103 cM) for hip ΔBMD was observed on chromosome 6q between DNA markers D6S1270 and D6S1021. Figure 3.5 shows the adjusted multipoint LOD scores across chromosome 6 for hip ΔBMD. This possible QTL signal was not observed in our previous cross-sectional linkage scan of baseline hip BMD (42), suggesting that this chromosomal region may affect ΔBMD but not peak BMD. No other significant or suggestive linkage signals were identified for hip, midpoint radius, ultradistal radius, spine or whole body ΔBMD.

Table 3.3. Residual heritability of change in BMD (45 to 65 years)

BMD site	n	h^2_r	SE	p-value	R^2
total hip	222	0.53	0.24	0.006	0.16
total spine	224	0.43	0.21	0.008	0.22
midpoind radius	227	0.44	0.23	0.020	0.08
ultradistal radius	228	0.33	0.23	0.055	0.07
whole body	210	0.24	0.17	0.055	0.10

Compared to the sample of all participants >45 years of age, results in the subset aged 45 to 65 were somewhat different. Point estimates for the residual heritability of ΔBMD at the hip (h^2_r = 0.53 vs. 0.44), ultradistal radius (h^2_r = 0.33 vs. 0.25), and whole body (h^2_r = 0.24 vs. 0.18) were greater in subset 45 to 65 years. Likewise, the linkage signal for hip ΔBMD on chromosome 6 was greater in the subset 45 to 65 years (maximum LOD = 2.5 vs. 1.7). The significance of the observed difference in heritability and linkage results between samples with and without inclusion of individuals >65 years was tested using a type of sensitivity analysis as described by Atwood et al. (125).

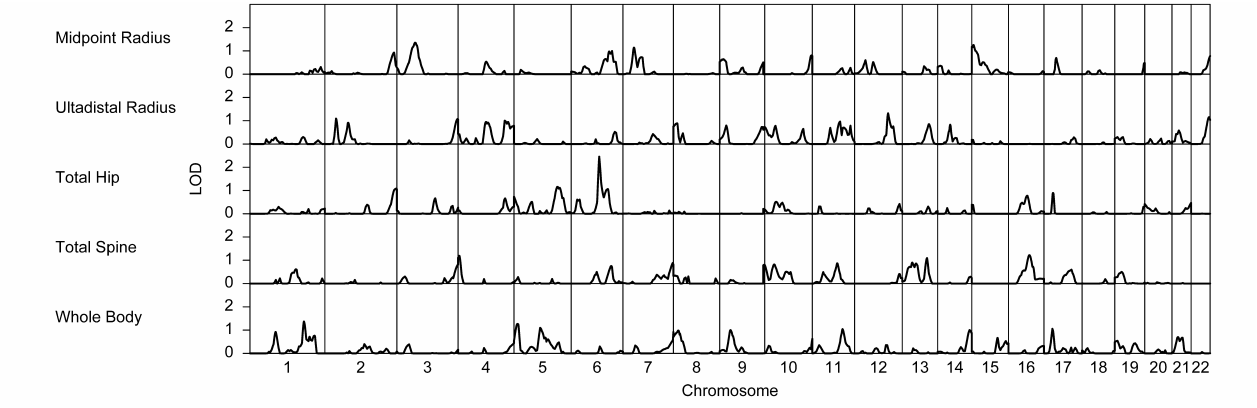

Figure 3.4. Multipoint LOD scores for change in BMD across chromosomes 1 to 22 (45 to 65 years).

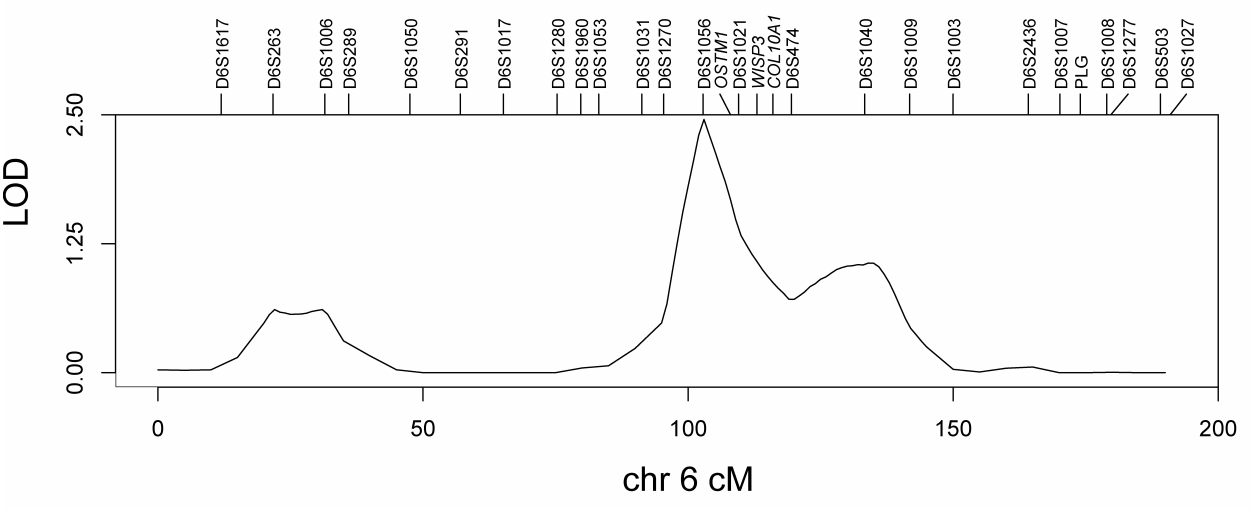

Figure 3.5. Chromosome 6 multipoint LOD score plot for change in hip BMD

In brief, to determine if these differences in heritability estimates and LOD scores are due only to the change in sample size, or alternatively, due to something intrinsic to the set of individuals >65 years, we re-ran our analyses for 1000 subsets in which 57 individuals (i.e. the difference in sample size) were removed at random from the total sample >45 years. This provides a sense of the null distribution for differences in heritability and maximum LOD scores due to changes in sample size alone. Based on these random subsets, for example, we found that point estimates of heritability for hip ΔBMD ranged from 0.0 to 0.75, and concluded that the difference in magnitude of heritability estimates observed with and without including participants >65 years was not significantly greater than expected due to the change in sample size ($p = 0.13$). In other words, inclusion or exclusion of participants >65 years does not affect heritability estimates. On the other hand, based on QTL scans of the 1000 random subsets, we found that, indeed, the inclusion of individuals >65 years reduced the observed linkage signal more than expected based solely on change in sample size ($p = 0.02$); that is, the observed QTL

result was in the upper 2% of the empirical distribution. This result suggests that heterogeneity may have been introduced by inclusion of the subset of individuals >65 years, specifically, which in turn diminished the evidence of linkage. Therefore, restricting our analyses to those aged 45 to 65 years may be an appropriate approach for dissecting the genetic factors influencing ΔBMD, although a large sample size would be required to achieve adequate power.

In the subset aged 45 to 65 years, the putative linkage signal on chromosome 6q, which exceeds the Lander and Kruglyak recommended threshold for "suggestive linkage" (99), is particularly exciting because it was not identified in our previously reported genome-wide linkage scan of cross-sectional BMD from this population (42), and has not been implicated as a QTL for peak BMD in other studies. Thus, this putative QTL may influence bone loss, and not peak bone mass, and may serve as a principal candidate region for higher-resolution analyses.

At least three biologically plausible candidate genes fall within our linkage peak on chromosome 6, among which is WNT1-inducible signaling pathway protein 3 (WISP3; located at chromosomal position 113 cM), a downstream member of the WNT pathway needed for proper skeletal growth. Several mutations in WISP3 are associated with progressive pseudorheumatoid arthropathy of childhood, an autosomal recessive skeletal disorder that results in destructive bone alterations and cartilage loss (126). A second gene in this region necessary for normal skeletal development is collagen type X alpha-1 (COL10A1; 116 cM). Specific mutations in COL10A1 cause Schmid-type metaphyseal chondrodysplasia (127), a form of short-limbed dwarfism with associated skeletal dysmorphisms. Because of its role in femoral neck development, possibly determining length, width, and neck-shaft angle (128), COL10A1 may also be involved in variation in hip ΔBMD with age. A third noteworthy gene in this region is osteopetrosis associated transmembrane protein 1 (OSTM1; 108 cM), an important regulator of

bone resorption (129). Possibly, one of these three candidate genes contributes to our linkage peak, although it should also be noted that there are currently 248 genes that map to this chromosomal region (i.e. between 85 cM to 120 cM on chromosome 6) that could also contribute to our linkage signal. To our knowledge, no QTLs for bone mineral density have been identified in homologous chromosomal regions in mouse (Mouse Genome Informatics 3.54; updated 06/23/2007) (130) or rat (Rat Genome Database; updated 06/11/2007) (131). Future exploration for genetic factors in this chromosomal region is needed, and may lead to new insights into the causes of common variation in rates of bone loss and management of bone health.

3.7 ACKNOWLEDGEMENTS

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4.0 QUANTITATIVE TRAIT LOCUS ON CHROMOSOME 1q INFLUENCES BONE LOSS IN YOUNG MEXICAN AMERICAN ADULTS

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4.1 ABSTRACT

We assessed heritability and performed genome-wide linkage analysis for early bone loss in a population-based sample of 327 Mexican American men and women from large pedigrees. Bone loss was heritable for the hip and forearm, with evidence of linkage to chromosomes 1q, 6q and 11p.

Introduction: Bone loss occurs as early as the third decade and its cumulative effect throughout adulthood may impact risk for osteoporosis in later life, however the genes and environmental factors influencing early bone loss are largely unknown. We investigated the role of genes on the rate of change in bone mineral density (BMD) in participants of the San Antonio Family Osteoporosis Study.

Materials and Methods: Rate of BMD change in 327 Mexican Americans (ages 25-45 years) from 32 extended pedigrees was calculated from DXA measurements at baseline and follow-up (5.6 years later). Family-based likelihood methods were used to estimate heritability and perform genome-wide linkage analysis (using a 7.6 cM map) for BMD change of the proximal femur, lumbar spine, and forearm.

Results: Rate of BMD change was significantly heritable for total hip, ultradistal radius and 33% ulna (h^2_r = 0.34, 0.34, 0.35, respectively, $p < 0.01$ for all), modestly heritable for femoral neck (h^2_r = 0.22, $p = 0.06$) and not heritable for spine BMD ($p = 0.5$). Covariates associated with BMD change included age, sex, baseline BMD, menopause, BMI, and interim BMI change, and accounted for 5% to 24% of phenotype variation. A significant QTL (LOD = 3.6) for femoral neck BMD change was observed on chromosome 1q23; suggestive signals were also observed for ulna BMD change on chromosome 11p14-15 (LOD = 2.5) and radius BMD change on chromosome 6q (LOD = 2.9).

Conclusions: We observed that early BMD loss was heritable, and performed one of the first linkage studies for BMD change. Linkage to chromosome 1q23 suggests this region may harbor one or more genes involved in regulating early bone loss of the femoral neck.

4.2 INTRODUCTION

Peak bone mass and rates of bone loss following menopause in women and later in life in men are commonly acknowledged to be important risk factors for osteoporosis. In fact, Hui et al., suggest that the contributions of peak bone mass acquisition and bone loss after menopause are roughly equal in determining bone mass in women 70 years of age (132). Over the past three decades, many of the factors that influence peak bone mass in adults, as well as bone loss among older men and women have been identified (3,6,85-89). In addition to environmental factors, numerous studies have documented that peak bone mineral density (BMD) is highly heritable (17,24-26,30,133) and many quantitative trait loci (QTLs, i.e. implicated chromosomal regions potentially harboring regulatory genes) have been reported (34,36-38,41-46,48-51,54,55,58,59,134,135). More recently, studies have clearly demonstrated that genetic factors regulate BMD change at several skeletal sites in women and older men. Specifically, the heritability of the femoral neck in premenopausal women (94), lumbar spine, forearm, and whole body (but not hip) in peri- and postmenopausal women (95), and lumbar spine, hip, and forearm (but not whole body) in older men and women (>45 years) (116) has been demonstrated. Studies to identify specific genes influencing peak bone mass and bone loss in older individuals are ongoing.

Although many studies have investigated genetic and environmental factors influencing peak bone mass and bone loss among older individuals, few studies have looked at the factors affecting early change in BMD among young adults, particularly the role of genetics on bone loss (26,93). While most cross-sectional studies report minimal bone loss among individuals <50 years of age (136), more recent longitudinal studies have revealed significant rates of bone loss occurring as early as the third decade in women (92,94,137-139) and men (92,140). Furthermore, results from cross-sectional and longitudinal studies indicate that the onset of trabecular bone loss may begin in early adulthood, whereas the onset of cortical bone loss is delayed until mid-life in women and later in life in men (17,92). Thus, bone health later in life may depend upon early bone loss, in addition to peak bone mass and advanced age-related bone loss. Furthermore, the importance of early bone loss with regard to later bone health may depend on the trabecular and cortical content of a particular skeletal site.

One study has reported that bone loss of the femoral neck is heritable in premenopausal Caucasian women (94), but to date, no study has assessed the genetic contribution to early bone loss at other skeletal sites or in men (younger than 45 years of age). Furthermore, no specific QTLs for longitudinal BMD change have been identified in any population.

As part of the San Antonio Family Osteoporosis Study, we sought to investigate the role of genetic factors on bone loss in younger individuals at a number of skeletal sites. Specifically, we have estimated the heritability of 5-year change in BMD in 327 Mexican American men and women (ages 25 to 45) from large multigenerational kinships, and performed genome-wide linkage analysis in search of QTLs influencing common variation in early bone loss.

4.3 MATERIALS AND METHODS

4.3.1 Recruitment and data collection

Data collection for baseline and follow-up phases of the San Antonio Family Osteoporosis Study have been fully described previously (30,116). In brief, participants from 34 multigenerational families were collected from a low-income neighborhood via a house-to-house recruitment protocol. Probands meeting eligibility criteria (i.e. aged 40 to 60 years and having large families in the San Antonio area) and all first, second, and third degree relatives and spouses were invited to participate irrespective of current health outcomes. Participating families represent a fundamentally unselected, population-based sample of Mexican American kinships, for which longitudinal data are available on 724 individuals.

Anthropometric, medical, and body composition data were collected during medical examinations at baseline (from 1997 to 2000) and follow-up (5.6 years later; from 2003 to 2006). Lifestyle, medical history, and reproductive history data were concomitantly assessed at both times via questionnaire. Approximately 81% of the original study participants were re-enrolled for follow-up.

The aim of the present study was to assess environmental and genetic influences on early bone loss; therefore analysis was performed in the subset of participants aged 25 to 45 years at baseline (n = 327). This cohort comprised participants from 32 kinships, including 1434 relative pairs (206 siblings, 96 avuncular relationships, 484 first cousins, and 648 other relationships).

BMD measurements of femoral neck, total hip, total lumbar spine (L1-L4), ultradistal radius, and 33% ulna (measured at 33% of total length from the distal end) were obtained by dual-energy x-ray absorptiometry (DXA) at both baseline and follow-up. Different sites of

radius and ulna are included because they represent different proportions of trabecular and cortical bone: ultradistal radius is largely trabecular, whereas 33% ulna is mostly cortical bone. During the interim between baseline and follow-up clinic visits, DXA equipment was upgraded from the Hologic 1500W model to the 4500W model (Hologic Inc., Bedford, MA), along with a software update to ensure comparability of scoring algorithms. Cross-calibration of absorptiometers showed near-perfect agreement on 10 test subjects (R^2 = 99.9%, 99.8%, and 99.9% for spine, total hip, and femoral neck sites, respectively; $p < 10^{-13}$ for all). No mean difference between absorptiometers was detected (paired t-test $p > 0.1$ for all sites). Precision of Hologic 1500W was 0.009 g/cm^2 for spine, 0.007 g/cm^2 for total hip, and 0.002 g/cm^2 for the manufacturer's spine phantom. Precision of Hologic 4500W was 0.006 g/cm^2 for spine, 0.007 g/cm^2 for hip, and 0.002 g/cm^2 for radius. For quality control, all DXA readings were performed by the same trained technician, measurement drift was prevented by calibrating equipment daily on the phantom, and baseline and follow-up scans were evaluated by the same reviewer ensuring comparability of regions of interest.

To prevent any unknown inter-machine differences from affecting our assessment of rates of BMD change, measurements of BMD were standardized (mean = 0, SD = 1) independently at baseline and follow-up. Annual change in standardized BMD was calculated as the difference divided by exact elapsed time between standardized baseline and follow-up measurements. This process retains the information of individual measurements relative to the sample, but not of the absolute magnitude of measurements, thus avoids potential unknown machine differences that could invalidate direct inter-machine comparisons. Rates of BMD change are expressed as percent of a standard deviation per year (%SD/year). Based on the precision of our DXA equipment, the least significant change (LSC; detectable with 95%

confidence) was ± 2.22 %SD/year for hip, ± 2.54 %SD/year for spine, and ± 1.53 %SD/ year for forearm. Observed rates of change less than the LSC values were not significantly different than zero (i.e. no observed change). Of the 327 participants included in this study, 65%, 73%, and 81% experienced significant rates of change in BMD (i.e. greater than the LSC) at the hip, spine, and forearm, respectively.

Covariates included in our analysis were sex, baseline age (years), site-specific baseline BMD (g/cm^2), baseline body mass index (BMI; kg/m^2), yearly interim change in BMI (kg/m^2/year; calculated as difference between baseline and follow-up measurements divided by the exact elapsed time), and interim entrance into menopause (yes or no; defined as surgical menopause or 1 or more years since most recent menstrual cycle). Covariate measurements were collected identically at baseline and follow-up clinic visits, as previously described (30).

4.3.2 Genotypes

Automated genotyping of the San Antonio Family Osteoporosis Study participants has previously been described (42). DNA from lymphocytes was amplified via polymerase chain reaction using fluorescently-tagged primers (MapPairs Human Screening Set Versions 6 and 8; Research Genetics, Huntsville, AL, USA) to detect repeat alleles at highly polymorphic microsatellite markers. Aliquots of amplified DNA were genotyped with Applied Biosystems Model 377 DNA Sequencers and analyzed with GeneScan and Genotyper DNA Fragment Analysis software (Perkin Elmer, Foster City, CA, USA). CRI-MAP (120) was used to assemble 460 microsatellite markers across chromosomes 1 to 22 into a genetic map, for which all marker positions were confirmed by deCODE (deCODE genetics, Reykjavik, Iceland). Mean inter-marker distance was 7.6 cM.

4.3.3 Statistical analyses

Distributions of BMD (at baseline and follow-up) and covariates were assessed, and outliers greater than 4 SD from trait means were excluded (0 to 3 observations removed per trait). Heritability of rate of BMD change was estimated in a variance components framework, which models phenotypic variance as a function of effects due to measured covariates, additive polygenic (based on average allele-sharing between relative pairs), and error components. The general form of this model is $y_i = \mu + \sum_{i=1}^{n} \beta_j X_{ij} + g_i + e_i$, where y_i is the rate of BMD change for the ith individual, μ is the sample mean rate of BMD change, X_{ij} is the jth covariate for the ith individual, β_j is the corresponding regression coefficient, g_i is the additive polygenic effect, and e_i is the residual error effect. Model parameters were estimated using pedigree-based maximum likelihood methods, from which residual heritability (h^2_r, i.e. the proportion of phenotype variance attributable to the additive genetic component after removing variation due to covariates) was estimated. The significance of covariate and heritable components were tested via the likelihood ratio test, which compares the likelihood of models including and excluding each component. This test follows a chi-squared distribution with 1 degree of freedom for testing covariates, and a 50:50 mixture of a point mass at zero and a chi-squared distribution with 1 degree of freedom for testing heritability. Only covariates with significant effects at $\alpha = 0.1$ were retained in final models for each skeletal site. The proportion of variance explained by covariates was determined by comparing models including and excluding retained covariates. Models of rates of BMD change for each skeletal site were restricted to participants with data on all retained covariates. Power to detect true heritability of 0.25, 0.35, and 0.45 was 70%, 85%, and 95%, respectively, at a significance threshold of $\alpha = 0.05$.

Multipoint linkage analysis was performed by extending the variance components model described above to include the effect of a theoretical QTL, σ_m^2, as a component of genetic variance. Multipoint identical-by-decent (IBD) probabilities across chromosomes 1 to 22 were estimated from genotype data of relatives via a Markov Chain Monte Carlo algorithm implemented in Loki (102). Maximum likelihood methods were used to estimate σ_m^2 based on the expected covariance due to IBD probabilities between relatives at each locus along the chromosomes. Significance of σ_m^2 was assessed by the likelihood ratio test, which compared the QTL model (i.e. including σ_m^2 as a component of variance) to the polygenic model (i.e. $\sigma_m^2 = 0$), and expressed as a logarithm of the odds (LOD) score ($\log_{10}$ of the likelihood ratio). This test follows a 50:50 mixed distribution of a zero point mass and a 1-degree of freedom chi-squared distribution. Empirical LOD score adjustment based on 10,000 simulated unlinked markers was used to guard against inflated LOD scores, which can occur due to deviations of the phenotype distribution from normality (104). Power to detect linkage was low: approximately 25% and 40% at thresholds of LOD = 2.0 and 1.5, respectively, for a QTL explaining 25% of variance in rates of BMD change.

Genetic modeling was performed in the set of all participants aged 25 to 45 years (n = 327) as well as the subset of age-eligible men and premenopausal women (n = 292). To assess whether differences in linkage results obtained from the total sample and premenopausal subset were due to diminished sample size (null hypothesis), or alternatively, due to inclusion of post-menopausal women, 100 random subsets of 292 individuals (sampled without regard to menopausal status) were used to generate an empirical distribution of the effect of reduced sample size (125). Genetic analyses were performed using the Sequential Oligogenetic Linkage

Analysis Routines (SOLAR) software (101); data management, summary statistics, outliers, and figures were done in R (R Foundation for Statistical Computing, Vienna, Austria).

4.4 RESULTS

Characteristics of the 327 participants included in this study are presented in Table 4.1. On average, adiposity in this sample was high (BMI > 30) in both men and women, and weight increased (0.82 kg/year in women and 0.45 kg/year in men) over 5.6 years of follow-up (range = 3.5 to 8.9 years). Distributions of yearly change in BMD (%SD/year) are shown in Figure 4.1. Change in BMD was calculated as the yearly difference between standardized (mean = 0, SD = 1) BMD measurements at baseline and follow-up in order to mitigate the effects of differences between baseline and follow-up measurements.

Genetic and environmental influences on BMD change for participants aged 25 to 45 years are shown in Table 4.2. Significant heritability of BMD change was observed for the total hip ($h^2_r = 0.34$, $p = 0.01$), radius ($h^2_r = 0.27$, $p = 0.03$), and ulna ($h^2_r = 0.35$, $p = 0.01$). Modest heritability was observed for the femoral neck ($h^2_r = 0.22$, $p = 0.06$). In contrast, BMD change of the total spine was not heritable. As expected, women who entered menopause during the interim between visits experienced significantly faster bone loss at the femoral neck, total hip, and total spine; however, this relationship was not observed for sites of the forearm (Table 4.3). Interestingly, women experienced slower rates of bone loss than did men (i.e. female sex was positively correlated with BMD change) for all sites except ulna. Bone loss increased with increasing age only at the forearm sites. The cumulative amount of variation attributable to

measured covariates differed greatly between skeletal sites, ranging from 5% for the ulna to 24% for the femoral neck.

Table 4.1. Mean (SD) population characteristics

variable	all	(SD)	women	(SD)	men	(SD)
sample size, n	325		210		115	
follow-up (years)	5.6	(0.7)	5.5	(0.7)	5.6	(0.7)
age (years)	34.4	(5.9)	34.6	(5.8)	34.2	(6.1)
anthropometrics						
height (cm)	162.2	(8.8)	157.4	(6.1)	170.8	(6.2)
weight (kg)	80.4	(20.7)	75.9	(19.2)	88.6	(20.8)
BMI (kg/m^2)	30.5	(7.0)	30.6	(7.4)	30.3	(6.3)
annual weight gain (kg/year)	0.69	(1.39)	0.82	(1.40)	0.45	(1.35)
annual change in BMI (kg/m^2/year)	0.27	(0.52)	0.33	(0.55)	0.15	(0.45)
medical						
diabetes (%)	10.7	-	10.4	-	11.2	-
pre-menopausal (%)	-	-	95.3	-	-	-
oral contraceptives (%)	-	-	20.9	-	-	-
interim entrance into menopause (%)	-	-	9.2	-	-	-
lifestyle						
alcohol consumption (%)	50.8	-	44.5	-	62.1	-
smoking history (%)	20.8	-	19.4	-	23.3	-
baseline BMD (g/cm^2)						
femoral neck	0.89	(0.13)	0.87	(0.12)	0.92	(0.13)
total hip	0.99	(0.15)	0.96	(0.13)	1.05	(0.15)
radius (ultradistal)	0.50	(0.07)	0.47	(0.05)	0.55	(0.06)
ulna (33%)	0.71	(0.08)	0.66	(0.05)	0.79	(0.06)
total spine	1.05	(0.12)	1.05	(0.12)	1.04	(0.13)
follow-up BMD (g/cm^2)						
femoral neck	0.87	(0.12)	0.86	(0.12)	0.89	(0.13)
total hip	1.01	(0.14)	0.99	(0.13)	1.06	(0.14)
radius (ultradistal)	0.49	(0.06)	0.47	(0.05)	0.53	(0.07)
ulna (33%)	0.77	(0.08)	0.72	(0.05)	0.85	(0.07)
total spine	1.03	(0.12)	1.04	(0.11)	1.01	(0.12)

Because menopause is widely acknowledged to have profound effects on bone loss, genetic and environmental influences on BMD change were also assessed after excluding 35 women who reported having undergone menopause during the years of follow-up. Heritability estimates in this subset were greater for the femoral neck ($h^2_r = 0.29$, $p = 0.05$) and total hip (h^2_r

= 0.49, p = 0.001), similar for the ulna (h^2_r = 0.39, p = 0.01) and spine (h^2_r = 0.0, p = 0.50), and lower for the radius (h^2_r = 0.25, p = 0.06). Standard errors and cumulative variance due to covariates (excluding the effect of menopause) were essentially unchanged.

Table 4.2. Residual heritability of BMD change

trait	n	h^2_r	SE	p-value
femoral neck	300	0.22	0.16	0.06
total hip	300	0.34	0.17	0.01
radius (ultradistal)	313	0.34	0.16	0.00
ulna (33%)	321	0.35	0.17	0.01
total spine	295	0.00	-	0.50

h^2_r = residual heritability

Table 4.3. Relationship between BMD change and covariates: beta-coefficients (p-value)

	femoral neck		total hip		radius (ultradistal)		ulna (33%)		total spine	
n	300		300		313		321		295	
R^2	0.24		0.16		0.14		0.05		0.14	
sex	1.9 [a]	(0.023)	2.2	(<0.001)	5.1	(<0.001)	-	(0.367)	3.8	(<0.001)
age	- [b]	(0.738)	-	(0.284)	-0.2	(0.002)	-0.2	(<0.001)	-	(0.489)
meno [c]	-4.4	(0.008)	-3.3	(0.009)	-	(0.699)	-	(0.310)	-5.5	(0.001)
BMI	5.1	(<0.001)	0.2	(<0.001)	-0.1	(0.097)	-0.1	(0.036)	-0.1	(0.045)
ΔBMI	0.6	(<0.001)	3.0	(<0.001)	1.4	(0.064)	-	(0.141)	-	(0.642)
BMD	-15.9	(<0.001)	-	(0.663)	14.9	(0.045)	-	(0.214)	-10.6	(0.002)

R^2 = proportion of variance due to covariates
beta-coefficients are interpreted as %SD/year per unit of covariate
[a] effect of female sex with respect to male sex
[b] only covariates with significant effects at α = 0.1 are included in genetic models
[c] meno = entrance into menopause during interim between baseline and follow;
ΔBMI = yearly change in BMI during interim (kg/m^2/year);
BMD = site-specific baseline BMD (g/cm^2)

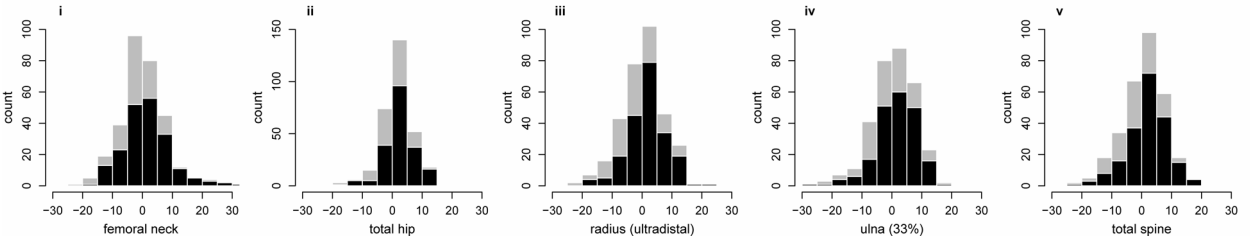

Figure 4.1. Distributions of change in BMD (%SD/year) for (i) femoral neck, (ii) total hip, (iii) radius, (iv) ulna, and (v) total spine. Grey bars represent the total sample (women + men); black bars represent women.

Figure 4.2 depicts whole genome multipoint linkage scans for BMD change of the femoral neck, total hip, radius, and ulna (but not spine because change at this site was not heritable). A significant QTL was observed on chromosome 1q23 at 151 cM for femoral neck BMD change (LOD = 3.6, unadjusted p = 0.00002, Figure 4.3). A suggestive QTL was observed on chromosome 11p14-15 at 31 cM for ulna BMD change (LOD = 2.5, unadjusted p = 0.0003). No other chromosomal regions exhibited evidence of linkage at LOD > 1.5 in the entire sample. In the subset of men and premenopausal women, linkage was diminished for the observed QTLs (LOD = 2.9 on chromosome 1, LOD = 2.1 on chromosome 11) and an additional suggestive QTL was identified for radius (LOD = 2.9 on chromosome 6q26-27 at 189 cM, unadjusted p = 0.00006) (results not shown).

To verify the robustness of our chromosome 1 QTL and determine whether the decrease in LOD score in the subset of men and premenopausal women was due to reduced sample size, or heterogeneity specific to postmenopausal women, we generated an empirical distribution for 100 subsets in which 35 (10.7%) individuals were excluded at random. The mean maximum LOD score across all leave-35-out subsets was 3.0 (at position 151 cM) which is not significantly different (p = 0.45) from our observed LOD = 2.9 in the subset of individuals that included only

men and pre-menopausal women. Thus, the observed decrease in evidence of linkage in the subset was likely due to a reduction in sample size alone and not evidence for heterogeneity.

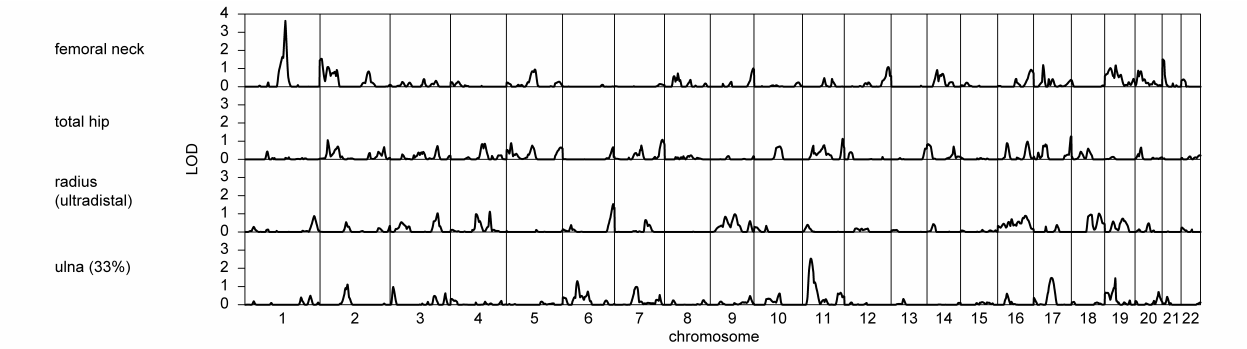

Figure 4.2. Genome-wide multipoint linkage scans

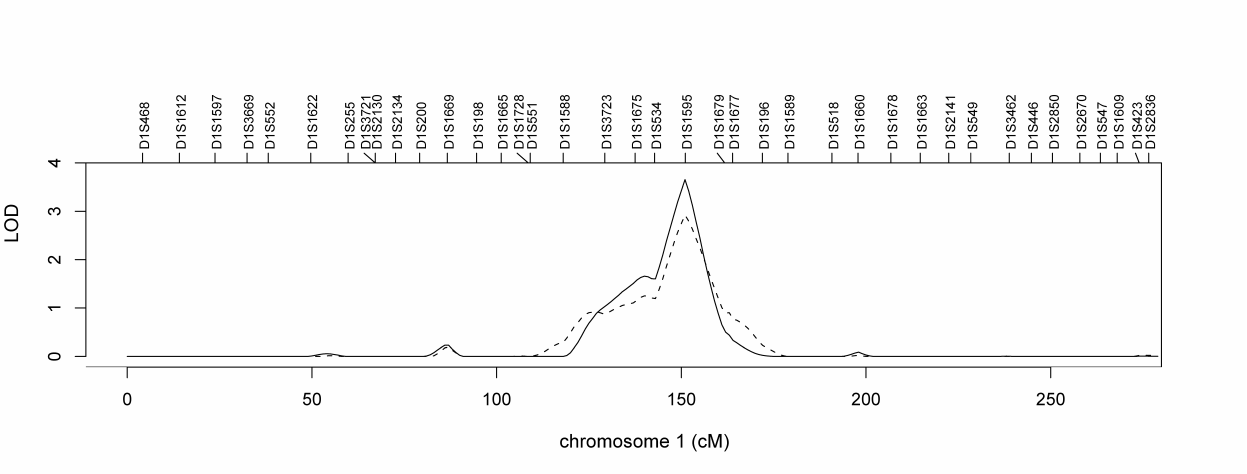

Figure 4.3. Multipoint LOD score profile for femoral neck BMD change on chromosome 1 in total sample (solid line) and subset of men and premenopausal women (dashed line)

4.5 DISCUSSION

Cross-sectional and longitudinal studies have shown that BMD declines in young men and women of European (92,138,140) and African ancestry (17), and that most of this change results from loss of trabecular bone density. One method by which to increase our understanding of the mechanisms influencing early bone loss is to determine that such longitudinal loss is heritable and subsequently identify the specific genes involved.

In this study we show substantial variation in the individual rates of early areal BMD loss at the femoral neck, total hip, radius, and ulna, and estimate that genetic factors are responsible for up to 35% of this variation. In contrast, we found that early bone loss at the lumbar spine was not heritable. Although our results include both men and women, they are consistent with those of Hui et al. (94) who reported heritability of femoral neck BMD in pre-menopausal women only. Our estimates of heritability of early bone loss are also similar, with a few exceptions, to those for bone loss among older individuals at several skeletal sites (95,116). Together, this body of evidence suggests that rates of BMD loss are heritable for many skeletal sites, in both men and women, young and old.

Our linkage scan yielded two putative QTLs influencing bone loss in young adults. The QTL for change in femoral hip BMD on chromosome 1q23 (LOD = 3.6) has been reported to influence variation in peak spine BMD in several (37,45,50,58), but not all (141), previous cross-sectional studies; specifically, this QTL was not observed in our Mexican American sample for either peak BMD (42) or BMD loss in older individuals (116). These possibly contradictory results could be explained by a QTL influencing BMD change, rather than peak BMD, in an age-specific manner. For example, linkage studies for peak BMD (37,45) and BMD change in young individuals are well-suited to detect a genetic regulator of early BMD change, whereas linkage

(42) and exclusion (141) analysis for BMD in mixed-aged samples, and linkage analysis for BMD change in older samples (116), are not.

The one-LOD unit support interval for the femoral neck QTL on chromosome 1q23 ranges from 146 to 155 cM (approximately 133 Mbp to 157 Mbp) in a gene-rich region containing 429 known or hypothetical genes. In particular, this region includes BGLAP/osteocalcin, which has well known effects on bone formation (111,142), and IL6R, for which previous studies have shown genetic association to BMD (143), and linkage to BMD and osteopenia (70). Additionally, a zinc/iron transporter (SLC39A1; mouse homolog expressed in osteoblasts of developing bone) (144), and 17 calcium binding proteins (S100A1-A7, -A7.1, -A7.2-A14, -A16) are contained in the support interval. One or more of these genes, or other linked loci, may contribute to our observed linkage signal.

We also obtained suggestive evidence for a QTL influencing early BMD change at the ulna on chromosome 11p14-15 (LOD = 2.5). Due to its position, this QTL is likely distinct from those detected in previous studies for peak BMD of the spine at 11q12-13, the LRP5 locus (41,46), or 11q23 (49). The one-LOD unit support interval for our suggestive QTL ranges from 22 to 42 cM (approximately 14 to 25 Mbp) covering 88 genes. Notable genes in this region are calcitonin alpha (CALCA), beta (CALCB), and pseudogene (CALCP). Calcitonin is a thyroid hormone regulating serum calcium levels, and has known inhibitory effects on the resorptive activity of osteoclasts (145). Calcitonin could also be involved in early BMD change and is a reasonable positional candidate gene for BMD change.

We speculate that because the femoral neck is comprised of both cortical and trabecular bone, and because growing evidence suggests that early bone loss occurs primarily in the latter (17,92), that observed linkage to chromosome 1q23 may be due to one or more genes affecting

trabecular bone loss. On the other hand, some early loss of cortical bone does occur (92). It is likely that the putative QTL on 11p14-15 affecting BMD change of 33% ulna, which is comprised almost entirely of cortical bone, is due to one or more genes influencing cortical loss. Though direct measurement of trabecular and cortical content are not available in this study, these hypotheses reflect recent findings in other populations (17,92), and are consistent with the largely uncorrelated linkage scans for different skeletal sites.

A major strength of the current study is the combined longitudinal and family-based study design. Our assessment of 5-year BMD change is better suited to elucidate the genetic effects of early bone loss than other cross-sectional linkage scans, and the inclusion of many types of relatives pairs within our extended families allows us to better model truly genetic effects on BMD change (as opposed to familial non-genetic effects, such household effects), than do studies using twins or sibships. These strengths notwithstanding, several limitations of the present study need also be addressed. First, while statistical power to detect heritability of BMD change was adequate, power to detect linkage at genome-wide significance was poor, and our sample size precluded testing for gene × sex or gene × environment interactions. Also, densitometry equipment was upgraded during the follow-up interim which may have introduced measurement bias; however, due to the independent standardization of baseline and follow-up measurements and excellent agreement observed in our cross-calibration experiment, any unknown effects of our equipment upgrade are probably minimal. Finally, DXA technology is inherently limited in its ability to detect BMD change because areal projection may not adequately account for the size (depth) of bone or possible change in bone size over time, and cannot measure trabecular and cortical content separately.

In conclusion, this study reports that early bone loss at several skeletal sites is heritable and similar in magnitude to the heritability of late bone loss. The QTLs for early bone loss that we detected on chromosomes 1q23 and, possibly 11p14-15 are among the first loci implicated in longitudinally-assessed BMD change, and may act as candidate regions for additional investigations into the genetic determinants of early bone loss. Identification of specific genes or pathways influencing early BMD change may lead to better understanding of bone health and possible therapeutic interventions for older populations at greater risk of osteoporosis.

5.0 REPEATED MEASUREMENTS CONFIRMS QUANTITATIVE TRAIT LOCI ON CHROMOSOMES 6q AND 13q INFLUENCE BONE MINERAL DENSITY OF THE HIP IN MEXICAN AMERICANS

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5.1 INTRODUCTION

Osteoporosis is a condition of profound medical and public health concern in the aging populations, worldwide. In the United States alone, more than 15% of women ages 50 to 59 and up to 70% of women over 80 years of age will be affected by this disease, contributing to approximately 350,000 hip fractures per year (3,146). In addition to mounting health care costs and significant morbidity associated with such fractures, including decreased mobility, the 1-year

mortality for those experiencing osteoporotic hip fracture is almost 25% (20). While other aspects of bone quality are also important, the primary indicator of bone strength and risk factor for osteoporotic fracture is bone mass (147)—usually quantified as bone mineral density across a projected area or volume—which has been shown to be highly heritable (27,28).

Many genome-wide linkage scans have been performed to identify chromosome regions associated with peak bone mineral density (BMD), yielding evidence for linkage to several chromosomal regions (58,122). In particular, possible quantitative trait loci (QTLs) influencing peak hip BMD have been mapped to chromosomes 1p (36,121), 2p (35,42), 2q (38,55), 3p (55), 3q (55), 4q (35), 5q (45), 6p (44), 6q (42), 7p (38,55), 12p (56),13q (42), 14q (48), 15q (48,56), 16q (38), 20p (51), 21q (44), and 22p (56). However, few of these candidate QTLs have been replicated in more than one study, which may be due to (i) population differences in the genetic determinants and/or gene × environment interactions affecting BMD, (ii) inadequate statistical power to detect QTLs, or (iii) false positive linkage signals (50). Because bone density is likely to be affected by a large number of genes, genetic heterogeneity—i.e. differences in the specific genes accounting for variation in BMD—between populations or cohorts is not surprising. However repeated studies in the same population may provide a means by which to distinguish false positives from QTLs that consistently influence BMD from one time point to another. On the other hand, BMD is truly a dynamic phenotype that changes greatly throughout the lifespan, and the genetic factors influencing BMD may be expressed differently across age ranges (24) and between men and women (25,42,43,50,55,56,58,135). Repeated measurements across longer periods of time, perhaps a decade or more, may serve as a means by which to investigate the age-specific genetic factors influencing BMD across the lifespan.

The San Antonio Family Osteoporosis Study (SAFOS) was designed to identify the environmental and genetic factors influencing BMD using data from 884 members of 34 multigenerational Mexican-American families. We have previously identified putative QTLs affecting hip BMD on chromosomes 2p, 6q and 13q in a subset of 664 individuals. Specifically, we reported a suggestive linkage signal for trochanter areal BMD (multipoint LOD = 2.27) on chromosome 6q in both sexes. In men (n = 259), we observed possible QTLs for femoral neck BMD on the telomeric region of chromsome 2p (LOD = 3.98), and for femoral neck and trochanter BMD on chromosome 13q (LOD = 2.51 and 3.46, respectively) (42). Subsequent to these analyses, genotype data for an additional 220 study participants were obtained, and follow-up BMD measurements were collected approximately 5.6 years after original measurements. Here we report an update and follow-up to our original genome-wide linkage scan including genome-wide linkage analysis of the total baseline sample, as well as new genome-wide linkage analysis of our follow-up sample. We have used repeated BMD measurements to confirm QTLs influencing common variation in BMD and provide insight into possible gene × age interactions.

5.2 METHODS

5.2.1 Recruitment and data collection

Participant recruitment and data collection for the SAFOS has previously been described in detail (30). In brief, a population-based sample of Mexican American probands, along with their spouses and all first, second, and third degree relatives of probands and spouses, were invited to participate in the study (n = 884, 34 families, 341 men, 543 women). Eligible probands had to

be between 40 and 60 years of age with large families in the San Antonio area. All available participants were enrolled without regard to prior medical history. Approximately 82% of the original SAFOS participants were available for a follow-up visit (265 men, 459 women), along with an additional 163 new recruits.

Areal BMD measurements of the proximal femur and whole body were assessed via dual-energy x-ray absorbtiometry (DXA) during a baseline examination (Hologic 1500W, Hologic, Inc., Bedford, MA) and again at a follow-up reexamination (Hologic 4500W) 3 to 8 years later. Cross-calibration of DXA scanners showed near-perfect agreement between machines ($R^2 > 99.8\%$, $p < 10^{-13}$ for femoral neck and total hip) (116).

As described in detail elsewhere (30,116), demographic, anthropometric, reproductive, medical and lifestyle variables were also measured during the baseline and follow-up exams. Based on our previous reports (30,116), we included the following covariates in this study: demographic covariates including age at examination, age^2, sex, age × sex, age^2 × sex; anthropometric covariates including body mass index (BMI; kg/m^2); reproductive covariates including menopausal status (defined as surgical menopause or >12 elapsed months without menstrual period), use of oral contraceptives; medical covariates including diabetes status (assessed by self-report, current use of antidiabetic medication, or glucose tolerance test); and lifestyle covariates including current alcohol consumption, current smoking, and supplemental calcium intake (yes/no as assessed by self-report).

Genotyping for SAFOS was carried out as previously described in detail (42). 460 highly polymorphic microsatellite markers across all autosomes were genotyped, and genetic maps were assembled via the program CRI-MAP (120) and confirmed from deCode (deCODE genetics, Reykjavik, Iceland). Mean inter-marker distance was 7.6 cM (Haldane).

5.2.2 Analytical methods

An autosome-wide QTL linkage scan for baseline hip BMD was previously reported for a subset of 664 study participants from 29 families (42). The purpose of the present analysis is to perform an updated linkage scan for hip and whole body BMD on all 884 participants in the baseline SAFOS sample, and to perform an initial linkage scan for hip and whole body BMD in the follow-up sample. Prior to genetic analyses, the distributions of BMD measurements and covariates were examined, and all observations in excess of 4 standard deviations from phenotype means were omitted (0 to 6 exclusions per phenotype). To reduce the suite of covariates (enumerated above) included in genetic analyses, we performed bidirectional stepwise multiple regression using Akaike information criterion. Covariates were removed or retained on a bone site-specific basis. Data management and preliminary manipulations were performed in the R environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria).

Variance components genetic modeling was employed to partition phenotype variation in BMD into components attributable to measured covariates, the additive genetic effects, and the residual error (101). Model parameters were estimated using pedigree-based maximum likelihood methods (i.e. while conditioning on the covariance structure of the family data). Statistical significance of each parameter was calculated by comparing the full (i.e. including all parameters) and reduced (i.e. constraining the parameter of interest) models via the likelihood ratio test, which follows a chi-squared distribution with one degree of freedom for individually testing covariates, and a 50:50 mixture of a point mass at zero and a 1-degree of freedom chi-squared distribution for testing genetic parameters. Environmental covariates that were statistically significant at a liberal threshold of alpha < 0.1 were retained in the genetic models to

reduce the amount of phenotypic variation not attributable to genes. The significance of the additive polygenic component was assessed by comparing maximized polygenic and sporadic phenotype models, which were simultaneously adjusted for covariates. The total phenotypic variation explained by covariates was determined by comparing models including and excluding all retained covariates. Genetic modeling was performed for the total sample and sex-specific sub-samples.

Because previous analyses identified sex-specific QTLs affecting hip BMD (42), we expanded this variance components modeling approach to test for evidence of sex-specific additive genetic variances in BMD. Modeling and hypothesis testing for sex-specific genetic effects has previously been described in detail (25). In brief, we allowed genetic variances to differ between men and women when modeling BMD in the total sample. Two specific hypotheses were then tested in this expanded model: (i) whether the magnitude of genetic variance (σ^2_G) was equal between men and women, and (ii) whether the inter-sex genetic correlation (ρ_G, i.e. extent to which the additive genetic effect on BMD in men correlates with that in women) (148) was equal to one. Unequal genetic variances would imply that genes account for more phenotypic variation in one sex than the other. Inter-sex genetic correlation significantly less than one would imply that different suites of genes are contributing to BMD variation in men and women.

Autosome-wide linkage analyses were performed within the same variance components framework, which modeled BMD variation as a function of effects due to a putative QTL component in addition to the previously enumerated components. Both two-point and multipoint linkage analyses were performed; multipoint identity-by-descent (IBD) probabilities were calculated via the Markov Chain Monte Carlo algorithm as implemented in Loki (102). The

significance of the QTL component at a given locus was determined by comparing the QTL model to the polygenic model, and is expressed as a LOD score.

To remedy the potential consequence of phenotype distribution (i.e. deviations from multivariate normality) on calculated LOD scores, 10,000 simulations of an unlinked marker were performed to determine the empirical LOD score distribution for each phenotype. Linear LOD score adjustments according to the empirical distribution were then applied to our findings for phenotypes showing inflated evidence of linkage (104). LOD score adjustments were not applied for phenotypes lacking inflated evidence of linkage. In other words, LOD score adjustments were conservatively applied to only reduce LOD scores, but not to increase them.

Because genetic effects on BMD may differ by sex and age groups, heritability assessment and linkage scans were performed for baseline and follow-up BMD measurements in the total sample and sex-specific subsets, as well as the subsets of individuals >45 and <45 years of age. Analyses were limited to individuals with available data for the BMD phenotype of interest and all retained covariates. Variance components modeling was carried out using the Sequential Oligogenetic Linkage Analysis Routines (SOLAR) software (101).

Table 5.1. Population characteristics at baseline and follow-up

	total sample				men				women			
	baseline		follow-up		baseline		follow-up		baseline		follow-up	
sample size	884		887 [a]		341		326 [b]		543		561 [c]	
demographics												
age (years)	42.8	(15.9)	47.4	(14.5)	42.1	(16.5)	46.3	(14.8)	43.2	(15.5)	48.0	(14.2)
anthropometrics												
height (cm)	162	(9.4)	162	(9.2)	170	(6.8)	171	(6.2)	157	(6.3)	157	(6.2)
weight (kg)	81.1	(20.0)	83.5	(20.8)	87.4	(19.7)	89.3	(20.2)	77.1	(19.2)	80.1	(20.5)
BMI (kg/m^2)	30.9	(7.0)	31.8	(7.3)	30.0	(6.2)	30.5	(6.2)	31.4	(7.5)	32.6	(7.7)
medical												
diabetes (%)	17.9		21.2		17.0		24.8		18.5		19.1	
reproductive												
pre-menopausal (%)									69.7		54.5	
oral contraceptives (%)									12.6		12.2	
lifestyle												
alcohol consumption (%)	41.7		43.4		59.9		59.1		30.2		34.2	
smoking (%)	21.5		18.7		30.1		25.6		16.1		14.6	
calcium supplment (%)	12.1		19.9		5.1		11.8		16.2		25.1	
bone mineral density (g/cm^2)												
femoral neck	0.87	(0.15)	0.85	(0.14)	0.90	(0.15)	0.88	(0.14)	0.84	(0.14)	0.83	(0.13)
trochanter	0.72	(0.12)	0.73	(0.13)	0.77	(0.12)	0.77	(0.12)	0.69	(0.12)	0.70	(0.12)
intertrochanter	1.18	(0.19)	1.18	(0.18)	1.25	(0.18)	1.25	(0.17)	1.13	(0.18)	1.15	(0.17)
total hip	0.99	(0.16)	0.99	(0.15)	1.06	(0.16)	1.05	(0.15)	0.95	(0.15)	0.96	(0.14)
whole body	1.12	(0.12)	1.11	(0.11)	1.20	(0.10)	1.17	(0.10)	1.08	(0.10)	1.08	(0.11)

[a] includes 724 returning and 163 new participants
[b] includes 265 returning and 61 new participants
[c] includes 459 returning and 102 new participants

5.3 RESULTS

Population characteristics for the baseline and follow-up samples are summarized in Table 5.1. Of the original sample of 884 participants, 724 (approximately 82%) were reenrolled for a follow-up visit along with an additional 163 new recruits (total follow-up n = 887). On average, this population was obese (BMI > 30) and experienced weight gain (2.4 kg) during the interim between exams. The prevalence of diabetes and use of calcium supplements also increased, whereas the number of current smokers decreased during the years between baseline and follow-up.

As expected, residual heritability was similar for baseline and follow-up measurements of BMD of the proximal femur and total body, ranging from 0.31 to 0.76 (Table 5.2). Measured environmental covariates accounted for between 10% and 47% of phenotypic variation. Environmental correlates of BMD in this population have previously been discussed in detail (30); in general, suites of covariates were similar for baseline and follow-up BMD and included age, age^2, sex, age × sex, age^2 × sex, BMI, and menopausal status, among others (Table 5.3). Together, genes and measured environmental covariates explained 64% to 86% of total phenotypic variation.

Table 5.2. Heritable, environmental, and residual error components of BMD variance at baseline and follow-up examinations for the total sample, men, women, subset >45 years of age, and subset <45 years of age

	baseline				follow-up			
	n	heritable	environ	error	n	heritable	environ	error
total sample								
femoral neck	829	0.35	0.39	0.26	741	0.41	0.31	0.28
trochanter	829	0.40	0.30	0.29	741	0.53	0.19	0.27
intertrochanter	829	0.33	0.38	0.28	809	0.39	0.32	0.29
total hip	829	0.33	0.40	0.27	808	0.43	0.34	0.23
whole body	823	0.31	0.46	0.23	784	0.48	0.32	0.20
men								
femoral neck	325	0.49	0.30	0.21	299	0.58	0.28	0.14
trochanter	325	0.60	0.11	0.28	299	0.64	0.15	0.21
intertrochanter	325	0.58	0.22	0.20	299	0.54	0.21	0.25
total hip	325	0.57	0.22	0.20	299	0.61	0.22	0.17
whole body	322	0.60	0.14	0.26	290	0.72	0.10	0.18
women								
femoral neck	504	0.42	0.40	0.19	442	0.35	0.32	0.33
trochanter	504	0.55	0.27	0.18	442	0.61	0.18	0.21
intertrochanter	504	0.37	0.36	0.28	510	0.47	0.30	0.24
total hip	504	0.39	0.37	0.24	509	0.45	0.35	0.20
whole body	501	0.33	0.37	0.30	494	0.56	0.33	0.11
>45 years of age								
femoral neck	339	0.49	0.31	0.19	273	0.32	0.32	0.36
trochanter	339	0.55	0.33	0.12	274	0.68	0.32	0.00
intertrochanter	339	0.41	0.42	0.16	274	0.36	0.38	0.26
total hip	339	0.44	0.43	0.13	274	0.44	0.39	0.17
whole body	333	0.35	0.47	0.18	259	0.39	0.38	0.23
<45 years of age								
femoral neck	490	0.38	0.32	0.31	394	0.41	0.27	0.31
trochanter	490	0.42	0.25	0.33	394	0.52	0.16	0.32
intertrochanter	490	0.50	0.33	0.17	394	0.44	0.25	0.32
total hip	490	0.44	0.35	0.21	394	0.46	0.25	0.29
whole body	490	0.53	0.36	0.11	389	0.76	0.12	0.12

Table 5.3. Environmental correlates of BMD at baseline and follow-up for total sample, men, women, subset >45 years, and subset <45 years:

Beta coefficients (p-value)

		sex	age	age^2	sex*age	sex*age^2	BMI	menopause	oral contra.	smoking
total sample										
femoral neck	baseline	-7.6 (6E-22)	-1.20 (5E-11)	0.009 (7E-06)	1.34 (6E-08)	-0.014 (3E-08)	0.88 (3E-40)	-	-	-
	follow-up	-4.1 (7E-05)	-0.36 (3E-19)	-	0.68 (1E-02)	-0.007 (2E-02)	0.85 (1E-36)	-3.59 (2E-02)	-2.68 (9E-02)	-
trochanter	baseline	-9.1 (6E-35)	-0.66 (7E-05)	0.006 (2E-03)	1.31 (9E-09)	-0.015 (8E-10)	0.63 (6E-26)	-	-	-
	follow-up	-5.9 (8E-09)	-	-	1.07 (6E-05)	-0.013 (5E-06)	0.70 (2E-26)	-3.02 (4E-02)	-2.84 (7E-02)	-
intertrochanter	baseline	-14.5 (3E-41)	-0.74 (2E-03)	0.006 (2E-02)	1.68 (4E-07)	-0.019 (5E-08)	1.39 (2E-53)	-	-	-
	follow-up	-12.2 (6E-30)	1.05 (4E-07)	-0.013 (1E-10)	-	-	1.14 (8E-38)	-	-	-
total hip	baseline	-12.8 (5E-46)	-0.94 (3E-06)	0.008 (2E-04)	1.54 (2E-08)	-0.017 (2E-09)	1.13 (1E-51)	-	-	-
	follow-up	-9.8 (2E-18)	-	-	1.21 (7E-08)	-0.014 (5E-12)	1.03 (2E-44)	-2.72 (9E-02)	-	-
whole body	baseline	-15.4 (2E-47)	-0.13 (7E-07)	-	0.75 (5E-09)	-0.009 (4E-11)	0.54 (5E-27)	-3.66 (1E-03)	-	-
	follow-up	-7.0 (2E-16)	-	-	0.87 (4E-07)	-0.011 (8E-12)	0.33 (1E-09)	-5.18 (3E-05)	-	-
men										
femoral neck	baseline	-	-1.26 (2E-09)	0.009 (2E-05)	-	-	0.91 (2E-13)	-	-	-
	follow-up	-	-0.35 (3E-15)	-	-	-	1.02 (1E-14)	-	-	-
trochanter	baseline	-	-0.75 (1E-04)	0.007 (1E-03)	-	-	0.63 (2E-08)	-	-	-
	follow-up	-	-	-0.001 (6E-03)	-	-	0.88 (2E-12)	-	-	-
intertrochanter	baseline	-	-0.88 (1E-03)	0.007 (1E-02)	-	-	1.61 (2E-21)	-	-	-
	follow-up	-	-	-0.002 (5E-04)	-	-	1.46 (1E-16)	-	-	-
total hip	baseline	-	-1.05 (5E-06)	0.009 (2E-04)	-	-	1.29 (1E-19)	-	-	-
	follow-up	-	-	-0.002 (4E-05)	-	-	1.27 (7E-18)	-	-	-
whole body	baseline	-	-0.13 (4E-05)	-	-	-	0.67 (9E-13)	-	-	-
	follow-up	-	-	-0.002 (6E-08)	-	-	0.39 (2E-04)	-	-	-
women										
femoral neck	baseline	-	-	-0.004 (4E-40)	-	-	0.91 (3E-33)	-	-	-
	follow-up	-	-	-0.003 (3E-07)	-	-	0.84 (6E-26)	-4.16 (5E-03)	-3.64 (1E-02)	-
trochanter	baseline	-	0.61 (7E-06)	-0.009 (7E-10)	-	-	0.64 (2E-20)	-	-	-
	follow-up	-	1.05 (6E-05)	-0.013 (5E-06)	-	-	0.64 (3E-16)	-3.54 (2E-02)	-3.27 (3E-02)	
intertrochanter	baseline	-	0.97 (2E-06)	-0.013 (5E-10)	-	-	1.29 (7E-34)	-	-	-
	follow-up	-	1.57 (9E-10)	-0.018 (2E-13)	-	-	1.03 (7E-23)	-	-	-
total hip	baseline	-	0.61 (2E-04)	-0.010 (3E-08)	-	-	1.07 (5E-35)	-	-	-
	follow-up	-	1.23 (1E-08)	-0.014 (7E-13)	-	-	0.93 (3E-28)	-2.96 (6E-02)	-	-
whole body	baseline	-	0.61 (4E-07)	-0.009 (3E-11)	-	-	0.49 (3E-17)	-3.93 (4E-04)	-	-
	follow-up	-	0.85 (2E-07)	-0.011 (4E-12)	-	-	0.32 (3E-07)	-5.30 (9E-06)	-	2.32 (3E-02)

Table 5.3. (continued)

		sex		age		age^2		sex*age		$sex*age^2$		BMI		menopause		calcium supplement	
>45 years of age																	
femoral neck	baseline	-6.7	(2E-08)	-		-		-		-0.005	(8E-18)	0.77	(8E-13)	-		-	
	follow-up	-		-		-0.003	(2E-09)	-		-		0.76	(4E-12)	-6.44	(9E-07)	-	
trochanter	baseline	-10.1	(2E-15)	-		-		-		-0.004	(4E-11)	0.57	(1E-08)	2.70	(9E-02)	-	
	follow-up	-9.4	(2E-12)	2.07	(1E-02)	-0.018	(4E-03)	-0.28	(4E-02)	-		0.66	(9E-10)	-		-	
intertrochanter	baseline	-15.2	(1E-19)	-2.42	(2E-02)	0.018	(3E-02)	2.77	(5E-02)	-0.027	(2E-02)	1.39	(3E-20)	-		-	
	follow-up	-14.2	(4E-13)	3.11	(1E-02)	-0.029	(2E-03)	-		-		1.14	(4E-13)	-		-	
total hip	baseline	-13.2	(7E-22)	-2.12	(2E-02)	0.016	(2E-02)	2.15	(7E-02)	-0.022	(2E-02)	1.09	(5E-19)	-		-	
	follow-up	-12.4	(1E-14)	2.35	(2E-02)	-0.022	(3E-03)	-		-		0.98	(2E-14)	-		-	
whole body	baseline	-16.8	(4E-37)	-		-		-		-0.004	(1E-09)	0.57	(3E-09)	4.50	(3E-03)	-	
	follow-up	-7.2	(1E-02)	1.50	(5E-02)	-0.015	(3E-02)	-		-		0.42	(4E-05)	-6.03	(8E-02)	-	
<45 years of age																	
femoral neck	baseline	-7.7	(4E-13)	-2.18	(1E-03)	0.022	(3E-02)	0.48	(6E-04)	-		0.96	(1E-29)	-		-	
	follow-up	-4.9	(1E-05)	-1.99	(2E-02)	0.021	(6E-02)	-		0.004	(6E-02)	0.95	(7E-24)	-		-4.66	(3E-02)
trochanter	baseline	-8.6	(4E-19)	-2.07	(6E-04)	0.026	(5E-03)	0.41	(9E-04)	-		0.66	(5E-19)	-		-	
	follow-up	-6.7	(1E-09)	-		-		-		-		0.69	(1E-14)	-		-	
intertrochanter	baseline	-13.8	(2E-23)	-2.05	(2E-02)	0.025	(7E-02)	-		0.008	(4E-03)	1.37	(2E-35)	-		-	
	follow-up	-11.3	(4E-13)	-		-		-		0.004	(1E-02)	1.09	(3E-18)	-		-5.86	(4E-02)
total hip	baseline	-12.3	(2E-25)	-2.22	(2E-03)	0.027	(2E-02)	0.45	(3E-03)	-		1.14	(2E-34)	-		-	
	follow-up	-10.0	(7E-15)	-		-		-		-		1.00	(2E-21)	-		-	
whole body	baseline	-11.2	(1E-47)	-		-0.003	(4E-03)	-		0.004	(4E-03)	0.57	(5E-24)	-		-	
	follow-up	-7.1	(6E-15)	-		-		-		-		0.32	(4E-06)	-		-2.93	(5E-02)

Interestingly, point estimates of heritability at both baseline and follow-up were generally greater in sex-specific subsets for both men and women than in the total sample. This observation may indicate that the suites of genes influencing variation in BMD only partially overlap between men and women; in other words, sex-specific genetic factors may be influencing BMD in this population. To further investigate this inference using the baseline data, we tested two specific hypotheses regarding sex-specific variances: (i) that genetic variance was equal between men and women (H_0: equal genetic variances), and (ii) that the inter-sex genetic correlation (i.e. the degree to which genes affecting BMD in men correlate with genes affecting BMD in women) is equal to one (H_0: genetic correlation = 1). Table 5.4 summarizes these hypothesis tests for BMD of the proximal femur and total body. We found modest evidence for sex-specific genes affecting BMD (i.e. $\rho_G < 1$) at the femoral neck ($p = 0.04$), intertrochanter ($p = 0.06$), and total hip ($p = 0.06$). There was no evidence of sex-specific genes influencing trochanter or total body BMD ($p = 0.11$ and 0.37, respectively), though such genes may exist, as our study may not have adequate power to reliably detect genetic correlations less than one. We found no evidence that the additive genetic variance differed in men and women (Table 5.4).

Table 5.4. Tests of sex-specific additive genetic effects on baseline BMD

BMD site	$\sigma_{G.men}$	$\sigma_{G.women}$	ρ_G	H_0: $\sigma_{G.men} = \sigma_{G.women}$ p-value	H_0: $\rho_G = 1$ p-value
femoral neck	9.88	8.49	0.75	0.33	0.04
trochanter	8.51	7.84	0.81	0.46	0.11
intertrochanter	12.08	10.77	0.79	0.91	0.06
total hip	10.28	9.33	0.78	0.60	0.06
whole body	7.16	5.70	0.96	0.97	0.37

$sigma_{G.men}$ = genetic standard deviation (i.e. square-root of genetic variance) in men;
$sigma_{G.women}$ = genetic standard deviation in women;
rho_G = inter-sex genetic correlation

Table 5.5. LOD score adjustment factors

	baseline	follow-up
total sample		
femoral neck	1.16	1.17
trochanter	1.08	1.16
intertrochanter	1.12	1.08
total hip	1.16	1.17
whole body	1.12	1.12
men		
femoral neck	1.37	1.43
trochanter	1.37	1.42
intertrochanter	1.51	1.41
total hip	1.52	1.65
whole body	1.29	1.61
women		
femoral neck	1.15	0.96
trochanter	1.10	0.99
intertrochanter	1.01	0.86
total hip	1.06	0.93
whole body	1.05	0.96
>45 years		
femoral neck	0.92	0.79
trochanter	1.09	0.95
intertrochanter	0.94	1.01
total hip	1.19	1.05
whole body	1.00	0.87
<45 years		
femoral neck	1.20	1.20
trochanter	1.08	1.13
intertrochanter	1.20	1.00
total hip	1.20	1.07
whole body	1.12	1.16

We next performed autosome-wide linkage analyses for baseline and follow-up BMD in the total sample, men, women and sub-samples >45 and <45 years of age, to search for overall, sex-specific, and age-specific loci implicated in BMD. Although a large number of linkage analyses are presented, the problem of multiple testing is greatly reduced due to the high genetic correlation between BMD at skeletal sites (rho_G = 0.73 to 0.98). Empirical LOD score adjustments were calculated from 10,000 simulations of a fully-informative unlinked marker, and are shown in Table 5.5. LOD score adjustment factors less than 1.0, indicating inflated

evidence of linkage, were applied to the findings presented herein. LOD score adjustments greater than 1.0 (i.e. indicating deflated evidence of linkage) were not applied; therefore, our results are likely conservative.

Autosome-wide multipoint linkage results are shown in Figure 5.1 for the total sample, men, and women, and Figure 5.2 for the subsets <45 years and >45 years of age. Putative QTLs (LOD > 2.0) at baseline and/or follow-up for proximal hip and whole body BMD were observed on several chromosomes (Table 5.6). Some of these chromosomal regions exhibited strong evidence of linkage at baseline or follow-up, but not both, suggesting that the QTL may be a false positive. For example, on chromosome 1q, linkage was observed for trochanter BMD in the total sample and subset <45 years of age (LOD = 2.22 for both) at follow-up, but not baseline (LOD = 0.59 and 1.58, respectively). Similarly, a likely false positive for femoral neck BMD located on chromosome 2p was observed in men at baseline (LOD = 3.04), but not follow-up (LOD = 1.05). Other non-replicated findings were observed on chromosome 10p (LOD = 2.45 and 2.32 at follow-up and 1.27 and 1.03 at baseline for intertrochanter and total hip BMD, respectively, in women), chromosome 7 (LOD = 2.71 at follow-up and 1.28 at baseline for intertrochanter BMD in the total sample), chromosome 11p (LOD = 3.37 at follow-up and 1.02 at baseline for whole body BMD in women), and chromosome 11q (LOD = 2.34 at follow-up and 0.40 at baseline for whole body BMD in women). One suggestive signal on chromosome 8 previously reported for baseline femoral neck BMD in a sub-sample of men (42) showed no evidence for linkage (LOD < 2.0) in the expanded baseline or follow-up samples.

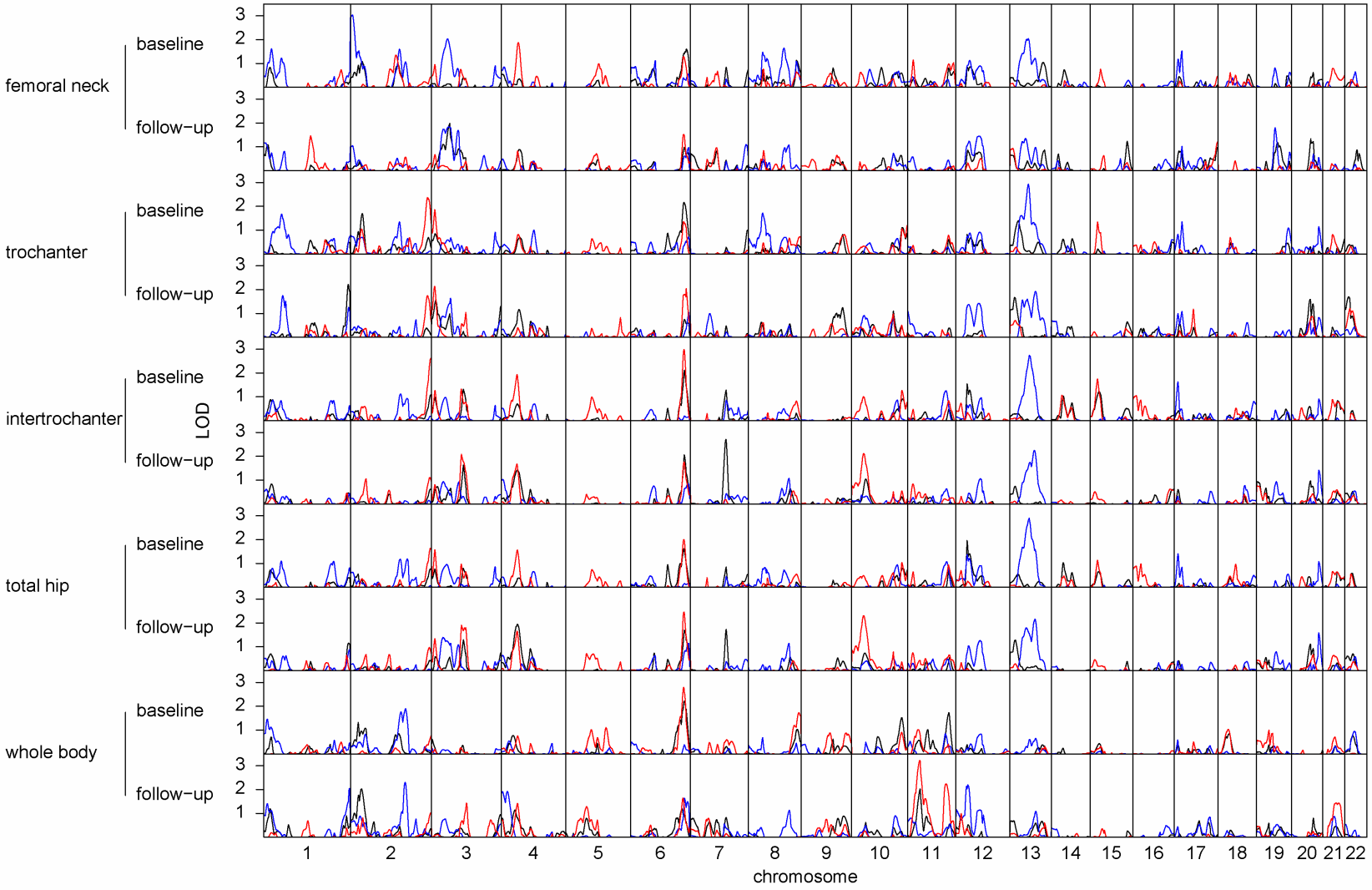

Figure 5.1. Multipoint linkage analysis of baseline and follow-up BMD in the total sample (black line), men (blue line), and women (red line)

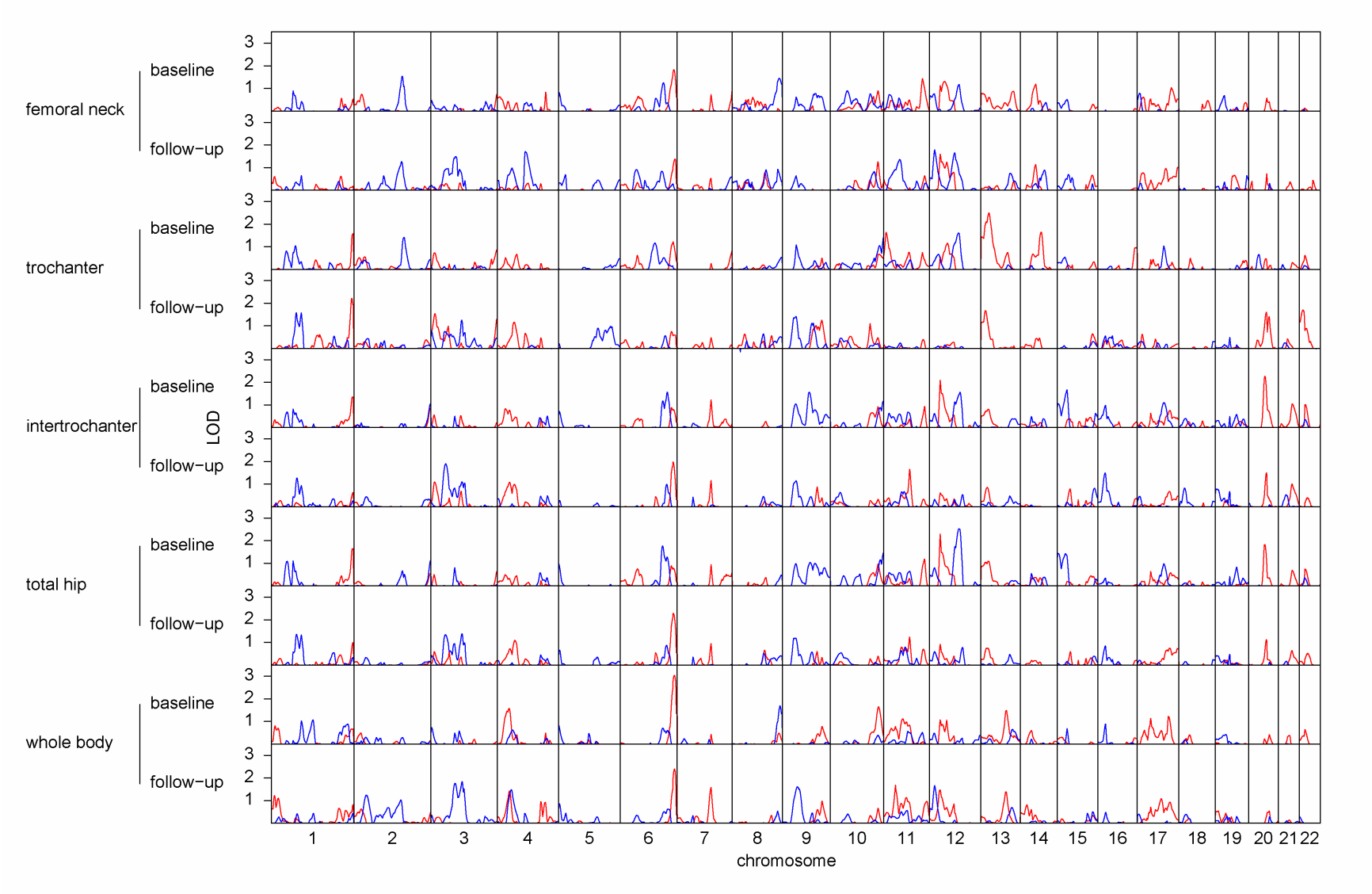

Figure 5.2. Multipoint linkage analysis of baseline and follow-up BMD in the subset <45 years (red) and >45 years (blue) of age

Table 5.6. Maximum LOD scores (>2.0) for chromosomes exhibiting linkage to baseline and/or follow-up BMD

chromosome/site	sample	baseline	follow-up	Kammerer et al. (42)
1				
trochanter	total	0.59	2.22	0.70
trochanter	<45 years	1.58	2.22	-
2				
femoral neck	men	3.04	1.05	3.98
trochanter	women	2.37	1.87	1.47
intertrochanter	women	2.62	1.10	-
whole body	men	1.90	2.30	-
3				
femoral neck	men	2.04	1.84	1.81
trochanter	women	1.86	2.29	0.66
intertrochanter	women	1.32	2.07	-
6				
trochanter	total	2.17	0.75	2.27
intertrochanter	total	**2.11**	**2.06**	-
intertrochanter	women	2.99	1.76	-
total hip	women	2.79	1.60	-
total hip	<45 years	0.89	2.29	-
whole body	total	2.22	1.20	
whole body	women	2.79	1.65	-
whole body	<45 years	**3.04**	**2.40**	-
7				
intertrochanter	total	1.28	2.71	-
8				
femoral neck	men	1.65	1.08	2.15
10				
intertrochanter	women	1.27	2.10	-
total hip	women	1.03	2.16	-
11				
whole body	women	1.02	3.24	-
whole body	women	0.40	2.25	-
12				
intertrochanter	<45 years	2.10	0.61	-
total hip	>45 years	2.53	0.78	-
total hip	<45 years	2.29	0.91	-
13				
femoral neck	men	2.04	1.35	2.51
trochanter	men	2.93	1.93	3.46
trochanter	<45 years	2.50	1.67	-
intertrochanter	men	**2.75**	**2.24**	-
total hip	men	**2.90**	**2.16**	-
20				
intertrochanter	<45 years	2.27	1.50	-

Bold indicates LOD > 2.0 for both baseline and follow-up samples

In contrast, some chromosomal regions exhibited consistent evidence of linkage at baseline and follow-up, or across multiple skeletal sites. The putative QTL on chromosome 6q, for example, showed suggestive evidence of linkage for trochanter, intertrochanter, total hip, and whole body BMD at baseline and/or follow-up in the total sample, women, and subset <45 years of age, with the greatest signal occurring for whole body BMD in the subset <45 years of age (LOD = 3.04 for baseline and 2.40 for follow-up). Likewise, chromosome 13q showed consistent evidence of linkage in men for all sites of the proximal femur, as well as in the subset <45 years for trochanter BMD. A broad region on chromosome 3p showed modest evidence of linkage in the total sample, men, and women, for various sites of the proximal femur, though no signal >2.0 was observed on chromosome 3p for both baseline and follow-up BMD at any site or in any sample; the cumulative evidence of linkage to this broad region hints that a QTL may or may not be present.

5.4 DISCUSSION

We conducted an autosome-wide linkage scan for hip and whole body BMD in the SAFOS population for an expanded sample of baseline measurements and a largely overlapping sample of follow-up measurements. Our motivations were to jointly determine if previously identified putative and suggestive QTLs for common variation in hip BMD could be re-substantiated after five years of follow-up and to search for additional QTLs that previously went undetected. We hypothesized that repeated measurements could help identify as possible false positive QTLs the regions showing linkage at one, but not both, time points.

Because phenotypes that vary across time may be due, in part, to genetic effects that vary across time, study designs that capture the dynamic nature of these phenotypes and that are better equipped to identify gene × age interactions are needed. Previous work has demonstrated the strengths of using repeated measurements in genetic studies. Some investigations compared linkage results from serial measurements of BMI to show that the effects of genes may vary across time (125,149), while others used longitudinal BMI measurements to form composite phenotypes reflecting trends over time (150,151). Other studies similarly used longitudinal measurements to investigate gene × age interactions affecting blood pressure (152) and change in lipoprotein risk factors over time (153). Taken together, these studies demonstrate the value of using longitudinal data in linkage analysis to help uncover genetic loci implicated in common disease. While the present study used repeated measurements to investigate genetic factors affecting areal BMD, others have used two-stage designs with expanded samples to confirm BMD linkage signals and increase power to detect new signals (40,49). Such designs may be particularly beneficial for dynamic phenotypes such as BMD.

Our analyses yielded evidence of two suggestive QTLs, on chromosomes 6q and 13q, which showed linkage in both baseline and follow-up samples, and for multiple skeletal sites. The 6q region (located at 150 to 170 Mbp on the physical map) contains 152 known or hypothesized genes, including a promising candidate for the observed signal, the estrogen receptor 1 gene (ESR1). ERα, the protein product of ESR1, is expressed in osteoblasts and osteoclasts, and may be involved in bone homeostasis (154,155). Association between polymorphisms in the ESR1 gene and BMD of the hip and spine has previously been reported in numerous populations (62,156,157), though it is unclear based on association studies whether the effect magnitude of ESR1 could account for the strong linkage peak observed on chromosome 6.

The implicated region on 13q (located at 40 to 75 Mbp on the physical map) contains 187 known or hypothesized genes, among which are two biologically plausible candidate genes: osteoprotegerin ligand (TNFSF11) and chondromodulin I (LECT1). TNFSF11 is expressed in skeletal regions undergoing ossification and remodeling, and thought to promote bone loss through its actions as an osteoclast differentiation and activation factor (158,159). LECT1 is a cartilage-specific glycoprotein that induces chondrocyte development. Mice lacking LECT1 exhibit increased BMD, reduced bone resorption (160), and vascular calcification (161). TNFSF11, LECT1, or other genes in this region may be responsible for the linkage signal observed on 13q in men.

Other genomic regions (i.e. on chromosomes 1q, 2p, 2q, 3p, 7q, 10p, 11p, 11q, 12, 20q) were implicated in BMD at one or more skeletal sites, but did not show consistency (at LOD > 2.0) between baseline and follow-up samples. One explanation for inconsistent results is spurious linkage at one time point. Such is the likely case for chromosome 2p in men, where the strong signal in the baseline sample (LOD = 3.04) was greatly diminished in the follow-up sample (LOD = 1.05). Other possible causes for non-replicated linkage results are small effect size (whereby power to detect linkage at a threshold of LOD = 2.0 is limited) and gene × age or menopause interaction (whereby the mean age difference between baseline and follow-up samples attenuates power to detect linkage). Such explanations are reasonable for modest signals demonstrating some degree of consistency, albeit not at the LOD = 2.0 threshold used in this study. For example, chromosome 2q shows suggestive linkage to trochanter BMD in women in the baseline sample (LOD = 2.37) and weak linkage in the follow-up sample (LOD = 1.87); thus, the joint interpretation of baseline and follow-up results is not entirely clear. These and similar results are consistent with the situation of a true QTL for which we have limited

power to detect linkage at LOD = 2.0. Moreover, recent work has shown that the variable rates of change in BMD over time among individuals are heritable in older (>45 years) (116), postmenopausal (95) and premenopausal (94) populations, but studies of peak BMD (including this one) assume sample-wide rates of change. Thus, genetic factors affecting BMD change may attenuate the apparent role of genes on peak BMD, and may do so differently depending on the age of the sample.

Prior to conducting our linkage analyses, we tested for evidence of sex-specific additive genetic effects influencing variation in hip and whole body BMD. Our findings of modest evidence for sex-specific genes are consistent with a growing body of previous work demonstrating varying degrees of sex-specificity for genetic regulation of BMD in humans (17,25,30,42,50,162,163) and mice (164). The lack of evidence for sex-specific genes influencing BMD at some anatomical bone sites may be due to insufficient power in our study to detect such effects. By modeling genetic variance independently in men and women, we greatly diminished the number of informative relative pairs because only same-sex relatives were utilized. Additionally, our analyses pooled subjects across a wide range of ages, thereby limiting our ability to detect possible sex-specific effects which differ across age cohorts. For example, sex-specific genes affecting a specific age cohort, such as bone acquisition in younger or bone loss in older individuals, are unlikely to be detected by our methods. Therefore, for anatomical sites where no sex-specificity was detected, the cumulative effect of sex-specific genes may simply be too small to distinguish from the effect of non-sex-specific genes. Interestingly, while we observed some evidence for sex-specific genes influencing BMD, we found no evidence that the magnitude of the total effect of genes differed between sexes. In other words, the impact of

genetics on BMD was similar between men and women, though the specific genes involved may differ.

Our linkage analyses for baseline and follow-up measurements of BMD are consistent with the hypothesis that some genes contribute to BMD in both sexes, whereas other genes are sex-specific. The putative QTL on chromosome 13q was observed only in men, suggesting an interaction between sex and the effects of genes in this region. The linkage signal on chromosome 6q for hip and whole body BMD, on the other hand, was observed for the total population, possibly indicating the presence of a putative QTL affecting BMD in both sexes. However, this QTL was also observed in women, but not in men, indicating that effects in women (who make up the greater part of the total sample) may be driving the observed signal in the combined sexes sample, whereas the effect in men may be more modest. Moreover, because this QTL affects whole body BMD, it may correspond to a gene with system-wide (rather than site-specific) affects on bone metabolism. ESR1, in this region, is likely to have system-wide effects, especially in women.

A major strength of this study is the repeated measurements of BMD which has allowed us to place greater confidence in replicated linkage signals, and cast doubt on non-replicated signals. However, defining and interpreting replication is problematic, requiring further investigation to distinguish false positives from situations where true QTLs are observed at a single time point. Our family data is sufficiently large to search for QTLs influencing BMD overall and within sex or age cohorts. However, we have insufficient power for testing other gene × environment interactions or for sub-dividing into more homogenous groups (e.g. post-menopausal women). Despite these limitations, and owing to the aforementioned strengths, this study has successfully confirmed our previously reported linkage signals on chromosome 6q and

13q, implicating these loci in the regulation of BMD in Mexican Americans. Further investigation of these regions, including inquiry into the candidate genes ESR1, TNFSF11, and LECT1 may yield better understanding of the biological processes governing common variation in BMD, and could lead to new insights for the risk assessment and treatment of osteoporosis.

6.0 LINKAGE ANALYSES FOR REPEATED MEASUREMENTS OF SPINE BONE MINERAL DENSITY IN MEXICAN AMERICANS

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6.1 INTRODUCTION

Vertebral fractures are exceedingly common: the lifetime risk of clinically diagnosed vertebral fracture is 16% in white women (5% for men), but because many fractures of the spine go undiagnosed, the actual rates are much higher (105). In a cohort of US women >50, the total prevalence of one or more vertebral fractures was 25% (165). Similar rates of vertebral fractures in postmenopausal women have been shown for other populations (105), reflecting the substantial public health impact of this condition. While not as devastating as hip fractures,

vertebral fractures can initially be very painful and lead to loss of height, kyphosis, poor posture, decreased mobility, and chronic pain. In addition to the physical detriment of spine fracture, dependency due to fracture and the related negative emotional impact may greatly decrease quality of life (105). As human longevity increases, the incidence of osteoporotic fractures and economic burden of treatment are also expected to rise (4), thus the problem of osteoporosis will continue to grow.

Like fractures of other skeletal sites, risk of vertebral fracture is due, in part, to bone strength, which is largely determined by bone mineral density (BMD). A number of environmental correlates of spine BMD have been described, including sex, age, height, weight, family history of fracture, menopause, estrogen use, and physical activity (30,105). In addition to environmental factors, genes also play a major role in determining spine BMD (17,22,24,25,30). Many linkage analyses have been performed to search for specific quantitative trait loci (QTLs, i.e. genomic regions influencing a continuous trait) affecting spine BMD in numerous populations. Chromosomal regions possibly harboring QTLs for spine BMD have been mapped to regions all over the genome, including: 1p (53), 1q (33,37,45,50,134), 2p (35,134), 3p (50,54,55), 3q (135), 4q (34), 5q (38), 6p (51), 7p (49,121), 11q (41,49), 12q (34,44), 13q (34,38,55), 14p (134), 14q (43), 15q (55), 16q (54), 17p (51,56), 18p (51,135), 20p (49,51), 20q (135), and 21q (50). Few of these QTLs have been replicated in more than one study, possibly due to false positives, genetic heterogeneity, differences in gene × environment interactions, or differences in demography, environmental risk factors, and ancestry across study samples. Analysis of repeated BMD measurements in the same population may aid in filtering possible false positive signals from those consistently observed across multiple time points. This approach has been employed in our study of the environmental and genetic causes of spine

BMD. Based on our previous results indicating differences in genetic architecture of bone loss among <45 and >45 years (Chapters 3 and 4), as well as differences between men and women (Chapter 5), we also performed analyses of spine BMD in these subsets.

6.2 METHODS

6.2.1 Recruitment

The San Antonio Family Osteoporosis Study (SAFOS) is a longitudinal study of the environmental and genetic determinants of bone strength-related phenotypes in an unselected sample of 34 multigenerational families of Mexican Americans. Recruitment and data collection has been described previously for baseline and follow-up phases of the study (30,116). Briefly, Mexican American probands aged 40 to 60 years with large families in the San Antonio area, and all adult first-, second-, and third-degree relatives of probands and spouses were invited to enroll in SAFOS without regard to prior medical history. At baseline, 853 participants (327 men and 526 women) with spine DXA measurements were recruited; at follow-up 3 to 8 years later (mean = 5.6 years), 859 participants with spine DXA measurements were recruited (686 returning and 173 new participants; 313 men and 546 women).

6.2.2 Data collection

BMD was assessed for lumbar vertebrae (L1 to L4) via dual-energy x-ray absorptiometery at baseline (Hologic 1500W, Hologic, Inc., Bedford, MA) and follow-up (Hologic 4500W).

Because measures of BMD of the four lumbar vertebrae (L1-L4) are highly correlated, we investigated total spine BMD, calculated as the total mineral content divided by total area of L1 to L4, in the current report. Cross-calibration of DXA scanners used at baseline and follow-up showed near-perfect agreement between machines (R^2 = 99.8%, $p < 10^{-13}$ for femoral neck and total hip) (116). Data for demographic, anthropological, reproductive, medical, and lifestyle variables were assessed via questionnaire at baseline and follow-up according to previously published protocols (30,116). In this study, the following covariates were considered: age (years), age^2, sex, sex × age, sex × age^2, BMI (kg/m^2), menopause (yes/no defined as >12 months since previous menstrual cycle or surgical menopause), use of oral contraceptives (yes/no), diabetes (yes/no), current smoking (yes/no), current alcohol consumption (yes/no), and supplemental calcium intake (yes/no). Spine BMD measurements or data on one or more covariates were not obtained for all participants. Genotyping for 460 highly polymorphic microsatellite markers and genetic map construction (mean resolution = 7.6 cM across chromosome 1 to 22) has been described previously (42).

6.2.3 Data analysis

Distributions of total spine BMD and aforementioned covariates were assessed, and all observations > 4 standard deviations from phenotype means were omitted (0 to 6 exclusions per phenotype). Statistically significant effects of covariates on baseline and follow-up spine BMD were assessed by bidirectional stepwise multiple regression analysis. Heritability of spine BMD was estimated using variance components methods, which deconstruct phenotype variance into the effects attributable to covariates, additive genetics (based on the expected sharing of relative pairs), and residual error (which includes non-measured environmental and non-additive genetic

effects). Maximum likelihood methods, while conditioning on the pedigree structure of the data, were used to estimate model parameters. Significance of heritability and covariates were determined via the likelihood ratio test by comparing models retaining and excluding the parameter being tested. The test statistic follows the chi-squared distribution with 1 degree of freedom for testing covariate effects, and a 50:50 mixed distribution of a point mass at zero and a chi-squared distribution with 1 degree of freedom for testing heritability. Covariates with significant effects at $\alpha = 0.1$ were retained in the model.

Multipoint linkage scans were performed by extending the variance components model to include the effect of a hypothetical QTL at an arbitrary genetic locus. Multipoint identity-by-descent (IBD) probabilities were estimated for all pairs of relatives at each genetic locus via a Markov Chain Monte Carlo algorithm as implemented in Loki (102). Pedigree-based maximum likelihood methods were used to estimate the QTL effect based on the covariance due to IBD probabilities between relatives across chromosomes 1 to 22. The significance of a hypothetical QTL at each locus was assessed by the likelihood ratio test comparing the QTL model to the polygenic model (i.e. no QTL effect), which follows a 50:50 mixed distribution with a point mass at zero and a chi-squared distribution with 1 degree of freedom. Significance of a hypothetical QTL at each locus is reported as a logarithm of the odds (LOD) score ($\log_{10}$ of the likelihood ratio). The possibility of inflated significance due to deviations from multivariate normality was checked by generating an empirical null distribution of 10,000 simulated, unlinked markers. Inflated LOD scores were adjusted based on the empirical distribution (however, deflated LOD scores were not adjusted, therefore results may be conservative).

Data analysis was performed for the total samples at baseline and follow-up, as well as subsets of men, women, participants <45 years, and participants >45 years of age. Analyses

were limited to individuals with data for BMD and all retained covariates, thus sample sizes for genetic models are considerably smaller than the total number of participants recruited. Genetic analyses were performed in the Sequential Oligogenetic Linkage Analysis Routines (SOLAR) software (101); data manipulations, outlier removal, stepwise regression, and figures were done in R (R Foundation for Statistical Computing, Vienna, Austria).

6.3 RESULTS

Descriptive statistics of the SAFOS population have been presented elsewhere (30,116). Mean age was 42.8 years at baseline and 47.5 years at follow-up (Table 6.1). On average, the sample was obese (BMI > 30) and gained weight (2.0 kg) during the years between medical exams. Likewise, prevalence of diabetes increased, and spine BMD decreased, between baseline and follow-up. Baseline spine BMD data were available for 853 of the SAFOS participants from 34 families (containing 651 parent-offspring pairs, 719 siblings, and 6,455 other relative pairs); follow-up spine BMD measurements were available for 859 individuals (573 parent-offspring pairs, 684 siblings, and 5,565 other relative pairs).

Table 6.1. Population characteristics: mean (SD)

	total sample				men				women			
	baseline		follow-up		baseline		follow-up		baseline		follow-up	
sample size	853		859		327		313		526		546	
demographics												
age (years)	42.8	(15.9)	47.5	(14.5)	42.2	(16.6)	46.4	(14.8)	43.2	(15.4)	48.1	(14.2)
anthropometrics												
height (cm)	162	(9.3)	162	(9.2)	170	(6.7)	171	(6.1)	157	(6.3)	157	(6.2)
weight (kg)	80.0	(18.6)	82.0	(18.5)	86.3	(18.2)	87.8	(18.1)	76.1	(17.8)	78.7	(17.9)
bmi (kg/m^2)	30.5	(6.4)	31.3	(6.4)	29.7	(5.7)	30.0	(5.4)	31.0	(6.8)	32.1	(6.7)
medical												
diabetes (%)	17.5		20.7		16.8		24.3		17.9		18.6	
reproductive												
pre-menopausal (%)									69.5		53.8	
oral contraceptives (%)									12.8		12.6	
lifestyle												
alcohol consumption (%)	41.9		44.5		60.2		61.1		30.5		35.0	
smoking (%)	21.6		19.2		30.0		26.6		16.4		14.9	
calcium supplement (%)	12.6		20.8		5.3		11.5		16.7		26.2	
BMD (g/cm^2)												
lumbar spine	1.03	(0.15)	1.01	(0.15)	1.06	(0.15)	1.04	(0.15)	1.01	(0.14)	1.00	(0.14)

Table 6.2. Environmental correlates of spine BMD: beta-coefficients (p-values)

	total sample		men		women		<45 years		>45 years	
baseline										
sex	-8.77	(9E-10)	-		-		-		-11.8	(1E-14)
age	-0.49	(1E-2)	-0.60	(6E-3)	0.69	(2E-5)	-		-1.76	(7E-3)
age^2	0.01	(1E-2)	0.01	(6E-3)	-0.01	(2E-10)	-		0.02	(3E-3)
sex × age	1.23	(5E-6)	-		-		-		-	
sex × age^2	-0.02	(7E-9)	-		-		-		-0.01	(2E-10)
BMI	0.62	(3E-18)	0.70	(2E-7)	0.60	(1E-13)	0.59	(3E-14)	0.71	(2E-7)
menopause	3.74	(2E-2)	-		3.58	(2E-2)	-			
follow-up										
sex	-		-		-		-		-13.2	(1E-12)
age	-0.55	(9E-2)	-		0.93	(4E-5)	-		-	
age^2	0.01	(6E-2)	-		-0.01	(2E-7)	-		-	
sex × age	1.59	(7E-5)	-		-		-		-0.53	(6E-6)
sex × age^2	-0.02	(1E-5)	-		-		-		-	
BMI	0.43	(8E-9)	0.52	(7E-4)	0.42	(7E-7)	0.37	(6E-5)	0.53	(3E-4)
menopause	-9.03	(3E-11)	-		-7.84	(2E-6)	-		-	
calcium suppl.	-2.75	(3E-2)	-		-2.44	(7E-2)	-		-	

beta-coefficients are interpreted as mg/cm^2 of BMD per unit covariate
sex effects are indicated for females with respect to males

Analyses revealed that suites of covariates with significant effects (at $\alpha < 0.1$) on spine BMD were similar for the baseline and follow-up samples (Table 6.2). In the total sample, the following determinants of baseline spine BMD accounted for 27% of phenotypic variation: sex, age, age^2, sex × age, sex × age^2, BMI, and menopause. In the follow-up sample, these covariates, with the addition of supplemental calcium intake and exclusion of sex, accounted for 19% of phenotypic variation. The residual heritability (h^2_r, i.e. the proportion of variance due genes after accounting for environmental covariates) of spine BMD was 0.54 and 0.61 in baseline and follow-up samples, respectively (Table 6.3). Altogether, genetic and environmental components of variance explained 66% and 69% of variation in spine BMD, respectively, in baseline and follow-up total samples.

Table 6.3. Heritable and environmental components of variation in spine BMD

					components of variance		
	n	h^2_r	(SE)	p-value	genetic	environ.	error
baseline							
total sample	828	0.54	(0.07)	1E-18	0.39	0.27	0.34
men	320	0.62	(0.12)	2E-09	0.57	0.08	0.35
women	508	0.70	(0.11)	1E-10	0.47	0.34	0.20
<45 years	486	0.78	(0.12)	3E-11	0.70	0.11	0.19
>45 years	342	0.74	(0.16)	4E-07	0.50	0.31	0.18
follow-up							
total sample	808	0.61	(0.08)	6E-18	0.49	0.19	0.31
men	295	0.67	(0.17)	2E-05	0.66	0.02	0.32
women	513	0.77	(0.10)	2E-12	0.57	0.25	0.17
<45 years	391	0.71	(0.14)	2E-07	0.69	0.04	0.27
>45 years	276	0.86	(0.18)	1E-05	0.69	0.20	0.12

h^2_r = residual heritability after removing the environmental component
environmental component of variance is due to covariates indicated in Table 6.2

In men, age, age^2, and BMI had significant effects on baseline spine BMD, whereas BMI alone had significant effects on follow-up spine BMD. These covariates explained only 8% and 2% of BMD variation, respectively. In women, age, age^2, BMI, menopause, and (in the follow-up sample only) supplemental calcium intake significantly influenced spine BMD, together accounting for 34% (in the baseline sample) and 25% (in the follow-up sample) of phenotypic variance. Spine BMD was highly heritable in men and women at both time points (h^2_r = 0.62 to 0.77; Table 6.3). Heritable and environmental components of variance explained 65% to 68% of phenotypic variation in men, and 80% to 83% in women. Significant heritability and covariate effects were also detected in the sub-samples aged <45 years and >45 years.

Table 6.4. Empirical LOD score adjustment factors

	baseline	follow-up
total sample	1.12	1.24
men	1.12	1.58
women	1.15	1.05
<45 years	1.27	1.32
>45 years	0.89	1.04

Multipoint linkage analyses, while simultaneously incorporating the effects of aforementioned covariates, were performed for baseline and follow-up spine BMD in the total sample, men, women, and subsets <45 and >45 years of age. To protect against inflated linkage signals due to deviations from multivariate normality (i.e. an assumption of the variance components methodology used to test for linkage), 10,000 unlinked markers were simulated to generate a null distribution. Empirical LOD score adjustment factors based on this null distribution are shown in Table 6.4. Adjustment factors <1.0 and >1.0 indicate inflated and deflated evidence of linkage, respectively. Based on these simulations, empirical LOD score

adjustment was applied to linkage results for baseline spine BMD in the subset >45 years of age (LOD adjustment factor = 0.89). LOD score adjustment factors >1.0 were not applied to findings presented herein, thus our linkage results may be conservative.

No QTLs meeting genome-wide significance were detected for spine BMD at baseline or follow-up in the total sample, men, women, or age-defined subsets. Figure 6.1 shows multipoint LOD scores across chromosomes 1 to 22 for all samples. Modest evidence of linkage (at a threshold of LOD > 2.0) was observed for follow-up spine BMD in the total sample (LOD = 2.5, position 53 cM) and men (LOD = 2.6, position 62 cM) on chromosome 19p13-q13. Two other loci yielded suggestive evidence of linkage: chromosome 2q12-14 for follow-up spine BMD in men (LOD = 2.1, position 174 cM) and chromosome 14q11 for baseline spine BMD in the subset >45 years of age (LOD = 2.1, position 39 cM). While a number of linkage scans were performed, the problem of multiple testing is partly alleviated due to the high genetic correlation between baseline and follow-up measurements of spine BMD (i.e. 99.77%).

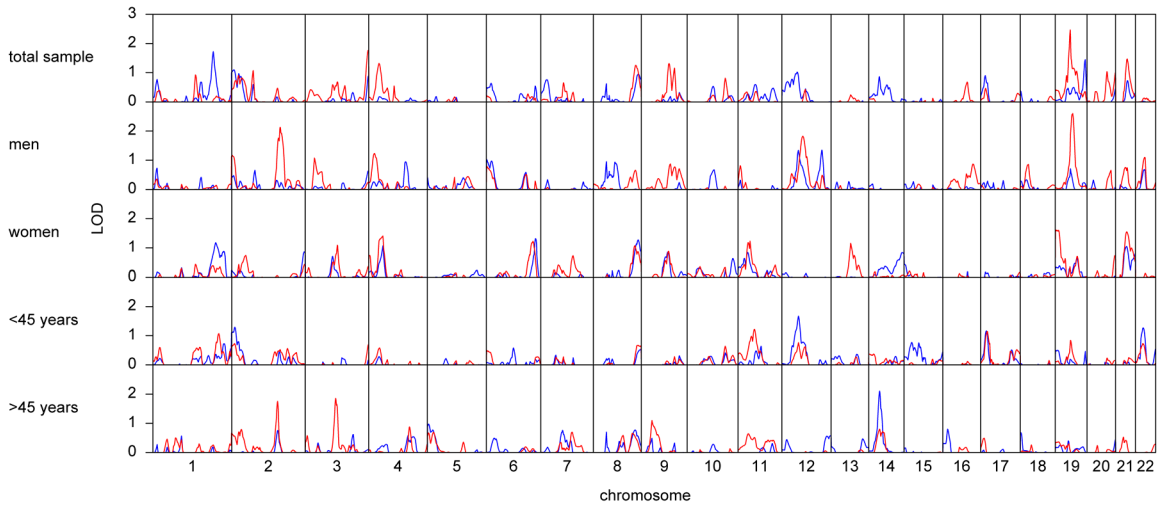

Figure 6.1. Multipoint LOD scores for baseline (blue) and follow-up (red) spine BMD across chromosomes 1 to 22

Table 6.5. Heritable and environmental components of variance in response of spine BMD to aging (i.e. follow-up spine BMD adjusted for baseline spine BMD)

					components of variance		
	n	h^2_r	SE	p-value	genetic	environ.	error
total sample	666	0.09	(0.06)	0.03	0.01	0.88	0.11
men	242	0.24	(0.14)	0.03	0.03	0.89	0.09
women	424	0.05	(0.09)	0.28	0.01	0.87	0.12
<45 years	392	0.09	(0.10)	0.17	0.01	0.86	0.12
>45 years	274	0.48	(0.19)	0.002	0.05	0.89	0.05

h^2_r = residual heritability after removing the environmental component

In order to investigate the role of genetic factors on the response of BMD to aging, we additionally modeled follow-up spine BMD while adjusting for baseline spine BMD (in addition to other significant covariates enumerated in Table 6.2). Table 6.5 shows results from this analysis, which may provide insight into the genetic basis of variation in the rates of change in BMD over time. When adjusted for baseline, follow-up spine BMD was weakly heritable in the total population ($h^2_r = 0.09$, $p = 0.03$), and moderately heritable in men ($h^2_r = 0.24$, $p = 0.03$) and in the subset >45 years of age ($h^2_r = 0.48$, $p = 0.002$). Estimates of heritability were not significantly different from zero in women ($h^2_r = 0.05$, $p = 0.28$) or the subet <45 years of age ($h^2_r = 0.09$, $p = 0.17$). Multipoint linkage scans for baseline-adjusted follow-up spine BMD in men and the subset >45 years did not yield evidence of any QTLs (maximum LOD = 1.44 on chromosome 17 for men; maximum LOD = 1.58 on chromosome 11 for the subset >45 years).

6.4 DISCUSSION

In this report we have estimated the heritable and environmental components of variation and performed autosome-wide multipoint linkage scans for spine BMD at two time points. There were two primary motivations for this study. The first was to search for environmental and genetic factors affecting spine BMD in one or both of the largely overlapping baseline and follow-up samples. We hypothesized that the concordance or discordance of findings in baseline and follow-up samples may aid in interpretation of the determinants of spine BMD by placing greater confidence in replicated results and casting doubt on non-replicated results. Moreover, in addition to the total samples at baseline and follow-up, analyses were carried out in the subsets of men, women, participants <45 years of age, and participants >45 years of age to find sex- and age-specific genetic effects on spine BMD. The second motivation for this study was to use repeated measures in the intersection of baseline and follow-up samples (i.e. the subset of participants with data at both time points) to assess the influence of genes on variation in 5.6-year response to aging. By modeling follow-up spine BMD, while adjusting for baseline BMD, we were able to evaluate the role of genetics for changes in spine BMD with age.

Similar to our previous reports on the genetic and envionrmental components of BMD in SAFOS men and women (30), these results confirm the high heritability of spine BMD for the total samples and age-specific subsets at baseline and follow-up, and indicate the strong effects of demographic variables (sex, age, and interactions), BMI, and menopause on spine BMD. We show strong concordance between the heritable and environmental components of variation in spine BMD at baseline and follow-up.

Linkage scans yielded no putative QTLs at genome-wide significance; the highest linkage signals occurred on chromosome 19p13-q13 in the total sample and in men (LOD = 2.5

and 2.6, respectively). This locus has not previously been reported to influence BMD of the spine (or any other skeletal region). The broad region under these overlapping linkage signals (19 Mbp to 49 Mbp) contains 433 genes including the transforming growth factor beta 1 (TGFB1) gene, which plays a regulatory role in the proliferation and differentiation of many cell types. Polymorphisms in the TGFB1 gene affect serum concentration levels (166), osteoclast formation, and resorptive activity (167), in addition to causing Camurati-Engelmann disease, a Mendelian skeletal disorder (168,169). TGFB1, or one or more other genes in this region may contribute to the suggestive linkage signals on chromosome 19. Two other suggestive QTLs (LOD > 2.0) were observed on chromosomes 2q12-14 (in men) and 14q11 (in the cohort >45 years).

None of the possible QTLs identified in this study were observed in both baseline and follow-up samples. Lack of replication between time points (in addition to modest evidence of linkage) hints that these suggestive QTLs may indeed be false positives. Other possibilities for the inconsistent findings between baseline and follow-up include small effect sizes such that power to detect linkage is limited or gene × age (or -menopause) interactions such that the mean difference in age between time points attenuates power to detect linkage.

The response of spine BMD to aging was modeled by adjusting follow-up measurements for baseline measurements. Significant heritability (with variable point estimates) of this response was observed in the total sample, men, and subset >45 years of age, though no chromosomal regions were implicated. This analysis is similar to and consistent with results showing heritability, but finding no evidence for specific QTLs, for annual change in spine BMD in this population (>45 years of age) (116).

Strengths of this investigation include the joint longitudinal and family-based study design, which has lent additional credence to replicated findings (i.e. heritable and environmental components of variance) and cast doubt on non-replicated findings (specific regions of linkage). Moreover, this approach has allowed us to assess the genetics of the response of spine BMD to aging, suggesting that genes may play a role in rate of change in spine BMD over time. In addition, we have evaluated the genetic and environmental determinants of spine BMD in a population of Mexican Americans, who are underrepresented in the osteoporosis literature. Concomitant with these strengths, however, are several limitations, including the possibility that the high prevalence of osteoarthritis and aortic calcification, especially in men, may have inflated densitometric measurements of spine BMD in some participants (113-115). Such noise may have reduced our power to detect linkage to spine BMD. Also, while our sample size was sufficient to model spine BMD in men, women, and age-specific subsets separately (albeit with loss of power with respect to the total sample), we did not have adequate numbers to further partition our sample into more homogenous groups (e.g. pre- or postmenopausal women). Likewise, we did not specifically test for gene × environment interactions affecting spine BMD. Additional work is warranted to further investigate the proposed QTLs for spine BMD and clarify the role of genes in the response of spine BMD to aging. Identification of the specific genes regulating spine BMD may provide important insights into our understanding of osteoporosis and may lead to better prevention and treatment of vertebral fractures.

7.0 CONCLUDING REMARKS

7.1 MOTIVATION

Considerable research has been done over the past four decades to identify both the genetic and environmental factors that influence bone health, especially peak areal BMD, and subsequent risk of developing osteoporosis. However, much less is known about the genetic and environmental factors that affect longitudinal change in BMD and its impact on bone health, especially among younger men and women. As part of this dissertation research, we investigated the genetic architecture of longitudinal change in BMD at several skeletal sites using data on large families of Mexican Americans, an understudied minority population.

7.2 SUMMARY OF MAJOR FINDINGS

The previous chapters of this dissertation detailed several important contributions of SAFOS to our understanding of the risk factors for osteoporosis. Specifically, we determined that BMD change varied over time and could be categorized into two phases: early adult bone loss (in participants <45 years of age) and later bone loss (in participants >45 years of age). Moreover, we showed that both of these phases of bone loss were significantly heritable at several skeletal sites, and identified environmental factors that influence bone loss. We performed the first

autosome-wide linkage analyses for longitudinally-assessed bone change traits and identified a possible QTL for early bone loss on chromosome 1q. By comparing genetic analyses of cross-sectional BMD at baseline and follow-up, we identified QTLs with consistent effects on peak BMD of the hip and showed that QTLs influencing peak areal BMD did not overlap with QTLs influencing bone change.

7.3 POINTS OF DISCUSSION

One of the major hypotheses examined in this body of work was that genetic heterogeneity for rates of BMD change may exist for different phases of bone metabolism; that is, we hypothesized that distinct genetic factors may explain part of the variation in rate of bone loss in younger, compared to older, participants. This idea was initially proposed based on the observed age-dependency of the trends in BMD and change in BMD in our sample (Figures 2.1 to 2.4) and in other studies (17,18,24,108,109), as well as genetic modeling showing age-specific genetic variance for rates of BMD change (Table 2.2). This hypothesis was further supported by our heritability and linkage analyses of BMD change in family members <45 years and >45 years of age (Chapter 3 and 4). For example, significant heritability was observed for BMD change of the hip and forearm in both age cohorts (h^2_r ranged from 0.31 to 0.44), and spine in the individuals <45 years of age (h^2_r = 0.42), whereas low heritability was observed in the total sample (h^2_r = 0.05 and $p > 0.05$ for skeletal sites of the hip and spine; $h^2_r < 0.20$ and $p < 0.05$ for sites of the forearm; results not shown). Likewise, significant and suggestive QTLs observed for early bone loss in the cohort <45 years of age were not observed in the cohort >45 years of age, and vice versa (Figure 3.2 versus Figure 4.2). For example, the strongest evidence of a QTL for

early bone loss was on chromosome 1q (LOD = 3.6), but there was no evidence of a QTL on chromosome 1 (maximum LOD = 0.80) for late bone loss. Although our sample sizes for early and late bone loss are small, these findings support the assertion that effects of genes influencing rate of BMD change may differ, at least in the magnitude of their effect, across the adult lifespan.

In contrast, genetic analyses of cross-sectional BMD (Chapters 5 and 6) did not differ between family members <45 years versus >45 years of age. Heritability of cross-sectional BMD was similar between age cohorts and the total sample, and age cohort-specific QTLs were not observed. These results are logical because current genetic models used in the analyses of cross-sectional BMD primarily reflect peak BMD acquisition. The effects of genetic (and environmental) factors influencing variation in peak BMD should act only during adolescence and young adulthood (or earlier). Therefore, the underlying determinants of peak BMD should not differ due to the age of the sample being analyzed as long as the analytical model used to recover peak BMD is accurate. Any observed differences in the determinants of cross-sectional BMD between different age cohorts in the same population (i.e. family members <45 and >45 years of age) would not truly be reflective of effects on peak BMD, but would instead be reflective of deviations in rates of change from the assumed age-trajectory of BMD. Overall, these results show that analysis of longitudinal data, rather than comparing age-specific analyses of cross-sectional data, may be a better approach for finding genes influencing change in BMD.

In addition to detecting possible QTLs that influence change in areal BMD, results of this project were also used to rank putative QTLs influencing peak areal BMD for future analyses. Although analysis of data collected on a largely overlapping set of individuals at a subsequent time point is not a true "replication", those chromosomal regions showing linkage in both baseline and follow-up samples marshal stronger evidence of a true QTL than do chromosomal

regions showing linkage at only one time point. Thus, among the three QTLs for peak hip BMD previously detected in a subset of the baseline sample, two of them (on chromosomes 6q and 13q) were also detected in the follow-up sample, and are given higher priority for future investigation.

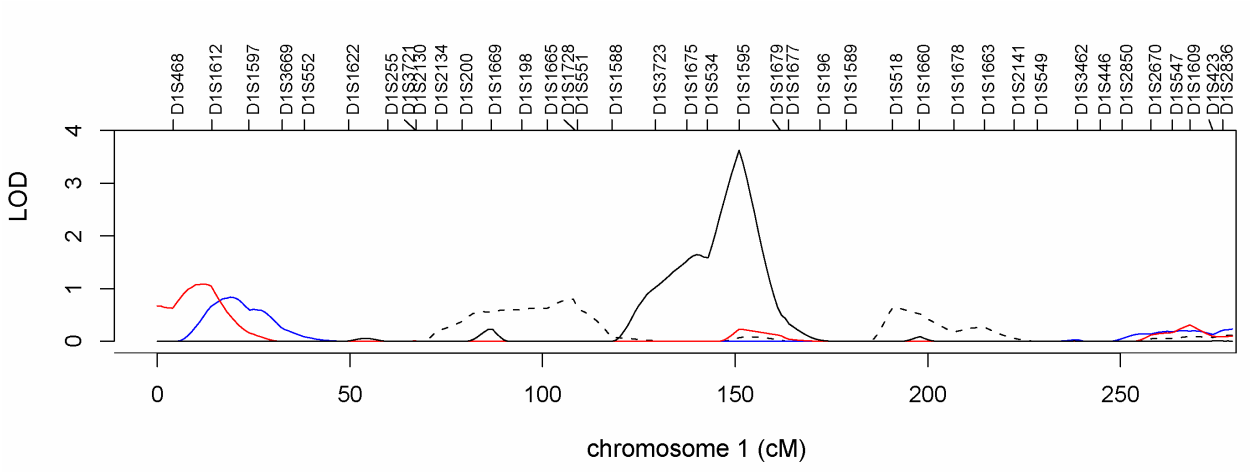

Figure 7.1. Multipoint LOD scores across chromosome 1 for: change in femoral neck BMD in the cohort <45 years of age (solid black line), change in total hip BMD in the cohort >45 years of age (dotted black line), baseline cross-sectional femoral neck BMD (blue line), and follow-up cross-sectional femoral neck BMD (red line).

One of our most exciting results was the identification of a statistically significant QTL on chromosome 1q influencing early femoral neck bone loss in the cohort <45 years of age. As described above, the QTL on chromosome 1q was not observed in analyses of BMD change for any skeletal site in participants >45 years of age, or in cross-sectional analyses of peak femoral neck BMD (or peak BMD of any other skeletal sites) at baseline or follow-up. Although the sample size is modest, these results indicate that while the QTL on chromosome 1q may influence early bone loss, it does not have detectable effects on late bone loss or peak BMD in

our population. Figure 7.1 compares multipoint LOD scores across chromosome 1 for BMD change in SAFOS participants <45 and >45 years, and cross-sectional BMD at baseline and follow-up. Likewise, the replicated QTLs for peak hip BMD on chromosomes 6q and 13q were not observed for BMD change of any skeletal sites in participants <45 or >45 years of age.

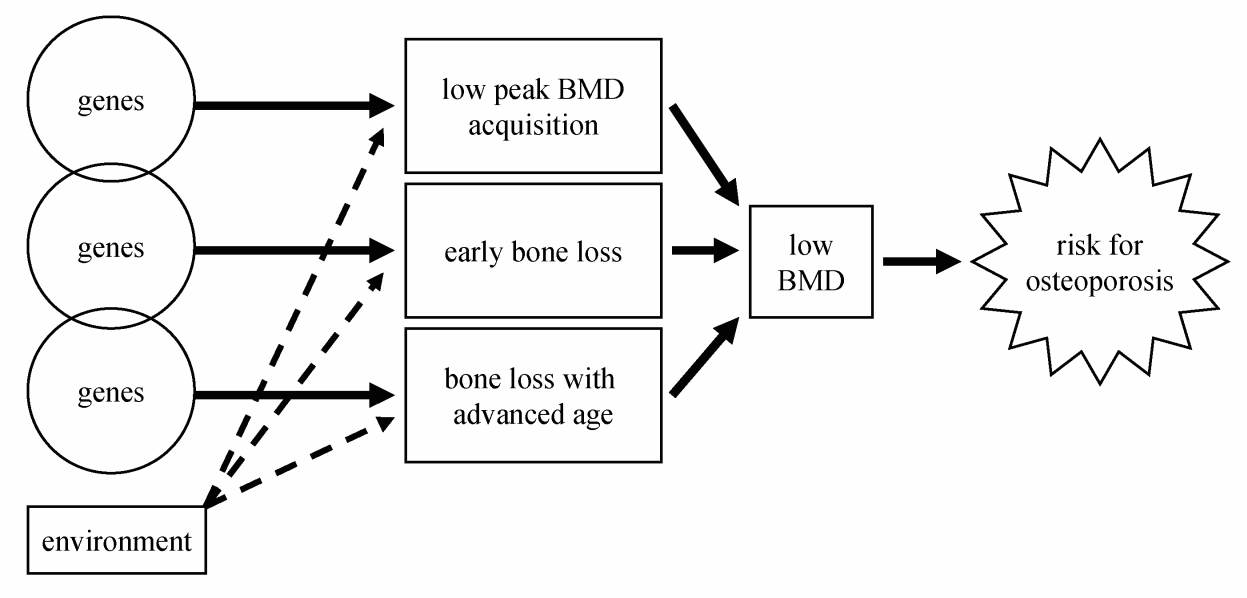

Figure 7.2. Risk model for osteoporosis

The investigations described in this dissertation were initiated under the risk model that osteoporosis and related heath outcomes are determined, in part, by the genes and environmental factors influencing peak BMD attainment in youth, and subsequent loss with age (Figure 1.1). Based on the results of this research, however, we have updated this risk model to include separate effects of genes and environmental factors on early-phase and late-phase bone loss in addition to the factors affecting peak BMD (Figure 7.2). Although this research has provided a better understanding of the relative effect of genes and environmental covariates on early- and

late-phase bone loss in both men and women, numerous questions still remain, including what specific genes and gene × environment interactions are involved, and whether the risk model differs between sexes or among different ethnic or geographic populations.

7.4 FUTURE CONSIDERATIONS

The natural history of bone metabolism over the lifespan, and the parallel effects on bone health and BMD, are not entirely understood. Thus, for studies of the underlying genetic and environmental architecture of BMD, such as this one, it is unclear what features of the data, including the timing of milestones or differences between sexes, age cohorts, populations, etc., may assist in devising the best way to analyze the data. We have used a relatively simple approach—analyzing family members <45 years and >45 years of age separately—to investigate factors affecting BMD change. Epidemiological work to more thoroughly characterize the changes over time in the architecture of BMD in Mexican Americans and other populations is needed to provide a better understanding of how these changes may impact different analytic approaches. For example, the choice of age boundary to describe the involutive change in age-dependency of BMD may differ by skeletal site or between sexes. This information could be used to better plan analyses to search for genetic and environmental determinants of BMD in the SAFOS population. Likewise, more advanced genetic models, which simultaneously consider the shared and unshared genetic determinants of phenotype variance among individuals younger and older than some age parameter could be developed to analyze BMD change.

Methods that jointly model longitudinal measurements across time, and simultaneously determine the shared and unshared effects of genes on baseline BMD, early bone loss, timing of

involution, and age-related bone loss, could also be developed. Rather than analyzing a single metric, longitudinal methods that can parameterize the trajectory of BMD over the adult lifespan may succeed in uncovering the roles of genes on bone metabolism. Genetic modeling of this sort would likely require multiple follow-up measurements. Reenrollment of SAFOS participants for collection of BMD data at additional follow-up time points could be used toward this end. Furthermore, an additional follow-up of SAFOS participants could also be used to increase sample sizes in age-specific cohorts for analyses parallel to those presented in this dissertation. For example, if individuals who were <45 years of age at baseline but >45 years at follow-up are recruited for one additional measurement (i.e. second follow-up), they would provide more observations, and could greatly increase power to detect possible QTLs affecting late-phase bone loss.

Different avenues of research could be used to further investigate the putative QTLs influencing peak BMD and early bone loss identified in the preceding chapters. In the upcoming months, genetic association of bone phenotypes will be performed for markers on the Illumina panel of 550K SNPs. Evidence for association to polymorphisms in our positional candidate genes and linkage regions will be carefully scrutinized. Genome-wide association analysis will be conducted in the search for other, novel, genetic factors affecting bone phenotypes. Functional analyses of any polymorphisms in genes showing association to bone phenotypes could also be pursued in vitro, and positive associations could be investigated in an independent population, such as the Amish.

7.5 PUBLIC HEALTH SIGNIFICANCE

As part of this research, we demonstrated that many of environmental risk factors for low BMD and bone loss observed in other populations are also important for osteoporotic risk in Mexican Americans, a population that is currently under-represented in the bone health literature. Importantly, we also showed early and age-related bone loss was heritable, and obtained strong evidence for a putative locus affecting early bone loss on chromosome 1q. Because osteoporosis is an enormous and growing public health problem, affecting approximately 30% of post-menopausal women (2), and even higher proportions of the elderly (3), understanding the environmental and genetic influences on bone health may lead to better prevention, risk assessment, and treatment of osteoporosis.

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