GENOME-WIDE ASSOCIATION STUDIES OF CHILDHOOD BONE HEALTH

by

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BS, University of Maryland, College Park, 2014

Submitted to the Graduate Faculty of

the Department of Human Genetics in the

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science and Master of Public Health

University of Pittsburgh

2016

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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April 5, 2016

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ABSTRACT

Osteoporosis is a major public health concern characterized by low bone mineral density (BMD) and deterioration of bone tissue, causing increased bone fragility and risk of fracture. Though current research has focused primarily on bone health in the elderly, early bone health, including peak BMD attainment, is a strong predictor of bone health later in life. Twin and family studies have demonstrated a strong genetic component in peak BMD, though the specific genes influencing variation in bone development are largely unknown. Moreover, the question of whether the genes influencing bone health during childhood are the same as those influencing bone health later in life is currently unknown. Therefore, to identify variants and genes implicated in childhood bone health, we performed separate genome-wide association studies (GWAS) for ten bone health phenotypes (bone mineral content [BMC] and BMD of the hip, spine, and head, BMC of the whole body, and four measures of hip geometry) in 296 Caucasian children aged 5 years (mean = 5.3) who were enrolled in the Iowa Bone Development Study. Linear regression while adjusting for sex, height, and weight was used to test 548,051 genetic polymorphisms and 7.4 million imputed variants for evidence of association. Genomic regions showing statistical association were scrutinized for relevant gene functions related to bone biology. Five genome-wide significant ($P \leq 5x10^{-8}$) and 30 suggestive ($P < 10^{-6}$) loci were identified in total. Implicated genes may represent significant roles in the converging pathways that regulate BMD, embryonic bone development, and bone remodeling. Furthermore, understanding the genetic determinants of

bone health during childhood may have implications across the lifespan. Though osteoporosis is usually viewed as an age-related disorder, risk of osteoporosis is impacted much earlier in life, including phases of bone mineral acquisition during youth. Therefore, the public health significance of this study is that identifying the genetic factors contributing to early skeletal health may ultimately lead to screening programs, which identify children with a genetic predisposition to bone disease. This allows for targeted interventions to optimize bone health in adolescence, promote management of bone health across the lifespan, and lower risk for osteoporosis later in life.

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PREFACE

I would like to take this opportunity to thank the individuals that helped me through this process. First, I would like to thank the dedicated members of my committee for their critiques, suggestions, and comments on my thesis. I thank my thesis advisor and research mentor, Dr. John Shaffer, for all of his patience, support, and encouragement over the past two years. He has been an amazing teacher and person to get to know!

I would like to thank Dr. Robin Grubs and Dr. Andrea Durst for their continual support and guidance throughout my time in the Genetic Counseling Program at the University of Pittsburgh. I would like to thank Dr. Candace Kammerer for her help and support in my Public Health Genetics training and in the development of my proposed public application of this study.

I would like to thank my family and friends for the words of advice, pep talks, and encouragement throughout my graduate training.

Lastly, I would like to thank my genetic counseling program classmates. They have become my Pittsburgh family and will be lifelong friends. Their kind support, many laughs, and great friendship has gotten me through this crazy process and I will forever cherish them.

1.0 INTRODUCTION

Skeletal health is instrumental to overall health and longevity. While some consider the skeleton as hard and lifeless, bones are in actuality living, growing tissue that have a major impact on overall health[1]. Bones have many functions in the body including providing structural support, protecting organs, fastening muscles and storing essential minerals[2,3]. Healthy bones are vital at every age and many factors affect bone health. However, poor bone health and bone disease represent a defining health problem of our aging society. This represents a serious public health threat due to adverse health consequences related to poor bone health, morbidity, and expensive treatment costs. Bone health is, therefore an important subject of research with the ultimate goal of improving disease prevention and treatment[4].

While we can take steps that optimize strong and healthy bones, understanding the molecular processes and regulation of bone growth and remodeling may allow us to implement new, targeted interventions. Bone is a rigid yet dynamic organ, which is constantly being molded, shaped and mended. Bone microarchitecture is determined by the physiological needs of the organism with the aim of maximum strength with minimal mass[5]. Throughout life, bone is continuously being built–up (bone formation) and broken down (bone resorption) as the result of many tightly regulated pathways. The current understanding of the contributors to bone health is that there are multiple factors including a strong genetic contribution[6,7]. While the knowledge of the specific genes that regulate bone growth has dramatically increased over the last decade, there

is still a large gap in our understanding of the genes responsible for bone variability and the interaction of multiple genes with each other and with environmental factors across the lifespan. The following sections present a comprehensive review of the current state of knowledge of the field.

1.1 BACKGROUND

1.1.1 Bone Function and Composition

Bone is a unique and highly specialized connective tissue that provides several essential functions for the body. The primary function of bone is to provide mechanical support and to provide a shield for the body's internal organs and tissues. Bone also acts a reservoir for minerals essential for many cellular processes of the body[8].

Morphologically, there are two forms of bone. First, accounting for approximately 80% of skeletal mass is cortical or compact bone[8]. Cortical bone is located in the shaft regions of long bones and is found primarily in the appendicular skeleton, such as the radius in the arm or the femur in the leg. Cortical bone provides the greatest mechanical and protective properties of bone because it consists of densely packed collagen fibrils, which causes cortical bone to be stiffer and stronger than trabecular bone, the other type of bone[7,9]. Trabecular or cancellous bone consists of a porous network, which allows this type of bone more flexibility. Trabecular bone is located in bone cortex and at the ends of long bones. This type of bone predominates the axial skeleton, such as the ribs and the spine. Due to its composition, the primary function of trabecular bone is metabolic functions, such as regulating calcium homeostasis[8].

The three major components of bone are the specialized bone cells, organic matrix, and mineral. Approximately 70% of mature bone consists of mineral[9]. The skeleton provides the major source of many essential minerals; these minerals are released when bone is broken down. The organic matrix is mostly comprised of calcium and phosphate crystals. Smaller amounts of magnesium, carbonate, and sodium are also found. The mineral crystals provide rigidity and the load-bearing strength of bones. Additionally, the distribution and size of mineral crystals within the bone matrix influence mechanical properties[8].

Approximately 30% of bone mass is organic matrix, of which the major constituent is type 1 collagen. Collagen fibrils play a role in providing the structural framework as well as the elasticity and flexibility of the skeleton. The remainder of organic materials are proteoglycans and non-collagen molecules, which are involved in bone mineralization and regulation of growth factors[8].

Lastly, approximately 2% of the mass of bone is specialized bone cells that maintain bone homeostasis. Genetic factors and molecular pathways tightly control the formation and differentiation of these specialized cells.

1.1.2 Bone Cells

There are four distinct sorts of specialized cells within bone. Osteoblasts, osteoclasts, and bone lining cells are found on the bone surface, while osteocytes are found implanted in the mineralized interior[4,8].

Osteoblasts are derived from multipotent mesenchymal stem cells and regulate bone growth and the production of bone extracellular matrix[2]. Mature osteoblasts are formed from bone lining cells and osteocytes. The differentiation and activity of osteoblasts negatively regulate

osteoclast activity. Osteoblasts also regulate mineralization of newly formed bone. From birth to adolescence, osteoblast activity and bone formation predominates, resulting in constant accumulation of skeletal mass[7].

Osteoclasts are specialized cells that are derived from the monocyte/macrophage hematopoietic lineage. These cells release acidic and lytic enzymes to degrade bone cells and induce bone resorption[5]. Degraded products such as collagen fragments and minerals are processed within the osteoclast and then released into circulation to be utilized in other cellular processes[2]. Osteoclasts are the key participant of bone remodeling during adult life. Most people in the U.S. lose approximately 0.5% of bone mass each year after age 40 due to the predominance of osteoclast activity[10]. Imbalances of remodeling can result in serious changes to the skeletal structure and potentially morbidity, affecting the lifespan. Most adult skeletal diseases are due to excessive osteoclastic activity; these diseases include osteoporosis, multiple myeloma, rheumatoid arthritis, and metastatic cancers[5].

Osteocytes are the most abundant and longest-lived bone cell type, and together they form the interconnecting network of bone cells that communicate through gap junctions[9]. Osteocytes are essentially osteoblasts that are encased in bone. Due to the signaling network of osteocytes, bone tissue is able to sense and transduce mechanical stress which is crucial to maintaining homeostasis[4]. Osteocytes can also detect micro-cracks, weight-bearing trauma, and changes in the hormonal state of bone in order to initiate the bone remodeling process[8].

Lastly, bone lining cells are flat, elongated bone cells. Bone lining cells are thought to be inactive though, if necessary can distinguish back into osteoblasts. The main function of bone lining cells is to act as a barrier for certain ions[8].

Altogether, these specialized bone cells are vital to the constant process of bone remodeling. Continuous remodeling is necessary in order to allow bones to adapt to changes in weight bearing load, and to repair the damage caused by recurrent microtraumas[7].

Bone homeostasis is essential for strong, healthy bones; however, the specialized bone cells must remain close to an equilibrium between bone production and bone resorption in order for bones to have the optimal strength and support the body. Bone mass varies based on the equilibrium between bone synthesis by osteoblasts and bone resorption by osteoclasts. Genetic factors that contribute to the regulation of this equilibrium, and more importantly genetic polymorphisms that cause variability in this equilibrium are essential to understand in order to identify individuals at risk for poorer bone health[6].

1.1.3 Bone Mineral Density and Content

Bone growth is characterized by longitudinal growth and the changes in skeletal size and shape[8]. Markers of bone growth and bone health include bone mineral content (BMC) and bone mineral density (BMD). BMC reflects the absolute amount of mineral in the selected bone[11]. BMD refers to the relative amount of bone mineral divided by projected area of the selected bone, or BMC divided by the area of the bone. Bone mass is accrued at different rates throughout the skeleton, which is why different sites demonstrate different rates of bone growth. BMD is a highly genetic trait that is commonly used for both the assessment of bone health in children and later in life, the diagnosis of osteoporosis[12]. Twin and family studies have demonstrated that the heritability of BMD and other factors of bone health, such as skeletal geometry, bone turnover, and ultrasound properties is high[13] Further, familial similarity of BMD and bone markers is higher during adolescence and early adulthood rather than later in life[14].

1.1.3.1 Bone Densitometry in Children and Adolescents

Bone tissue is influenced by genetic, metabolic, and behavioral elements. A number of disorders are known to affect bone development and bone mineral accrual in children, resulting in poor growth and/ or delayed bone maturation[15,16]. Tools to assess bone health is needed to identify pediatric patients at risk for poor bone health or at risk of osteoporosis due to low BMD[17]. Dual x-ray absorptiometry (DXA) is the most commonly utilized technique for determining BMD and BMC. DXA is ideal for pediatric measurements because it is widely available, has a rapid scan time, and low radiation exposure[17,18]. DXA is an effective, noninvasive and quantitative method for assessing the risk of fractures[9]. Reference curves for BMD and BMC at several body sites have been reported for children ages 5 -20 years from the Bone Mineral Density in Childhood Study (BMDCS), a national study of childhood standardized DXA measurements[17].

1.1.4 Peak Bone Mass

Bone growth during childhood and adolescence is a strong predictor of the lifetime risk of bone disease[8]. Peak bone mass, which is reached by early adulthood, is the point of bone homeostasis in which bone formation predominates shifts to the period in which bone resorption predominates. On average, peak bone mass is achieved around the age of 25 years and is the key determinant of the lifetime risk of osteoporosis[4]. Along with its impact on growth, puberty plays a fundamental role in the acquisition of bone mass[8]. Skeletal mass approximately doubles during the period between onset of puberty and young adulthood [8]. Puberty is a critical time in which the amount of bone mass accumulated is equal to the amount of bone typically lost throughout life. The current accepted belief is that the higher the peak bone mass achieved during adolescence, the less impact bone resorption at older ages has before and individuals begins experiencing compromised skeletal

function and risk of osteoporosis and fractures. Because of this, optimizing peak bone mass is important to lower the risk of osteoporosis and fractures. Interventions that aim to optimize peak bone mass are the most beneficial as lifetime risk of fracture incidence is reduced by an estimated 40% for each gain of 5% (0.5 Standard Deviation) in peak bone mass[19]. Up to 80% of the variation in peak bone mass has been accredited to genetic factors[16]. Therefore, programs that could identify individuals with genetic risk factors to low peak bone mass as well as implement effective intervention strategies to maximize peak bone mass could prove to be a successful osteoporosis prevention initiative.

1.1.5 Genetic Regulators of Bone Biology

Studies over the past forty years have repeatedly established a strong genetic component to bone mass and development[20]. Genetic factors have been recognized as having an essential role in the pathogenesis of osteoporosis due to the variance in peak bone mass as well as other key indices of bone health including osteoporotic fracture risk, bone geometry, bone turnover, muscle strength, and body mass index[13]. Studies have estimated a heritability of BMD as high as 92%[21,22] as well as a 50-80% heritability of other markers of bone health, including bone turnover and bone geometry[8,21]. Several approaches have been implemented to distinguish candidate genes; these approaches include association studies, linkage analysis, model organisms, and twin studies[13,21,23].

1.1.5.1 Candidate Genes Implicated in Previous Literature

Many genes have been suggested as likely candidate genes for the regulation of bone health through several techniques. Studies have demonstrated genes that regulate bone homeostasis have

site specific and gender specific effects[21]. The most widely studied genes are listed in the table below[24,25].

Table 1: Candidate Genes of Bone Development from Previous Literature

Candidate Gene	Proposed Function
RANK/RANKL	RANK/RANKL signals the formation of osteoclast precursors, activation, and survival in normal bone remodeling [5,26,27]. Mice deficient in RANKL and RANK develop osteopetrosis because of the inability to form osteoclasts[28,29].
OPG	This gene acts as a negative regulator of bone resorption. Overexpression blocks osteoclast formation inducing osteopetrosis. Gene knockout results in enhanced bone remodeling and bone loss, resulting in osteoporosis[5].
COL1A1/2	Encode matrix proteins. The expression of these genes and the balance of collagen fibrils are essential for proper formation of bone. Poor collagen quality results in reduced bone strength. Mutations in the *COL1A1* gene results in osteogenesis imperfect, a genetic condition with a phenotype of extremely severe osteoporosis[8,20,21].
LRP5	A receptor for canonical Wnt signaling. Wnt signaling is involved in processes including apoptosis, limb development, and osteoblast and chondrocyte differentiation. Knockout mice have shown low bone mass is a result of decreased osteoblast proliferation[13,30].
TGF-β1	Abundant in bone matrix, the released TGF-B1 protein is an essential controller of osteoblast proliferation and differentiation. Gene knockout results in osteopenic phenotype[31].
Osterix	Transcriptional regulator expressed in chondrocytes and in osteoblasts. Targeted inactivation of this gene led to the complete absence of bone synthesis throughout the skeletal and a loss of most markers of bone differentiation[20].
VDR	The first candidate gene to be investigated in the osteoporosis field, the *VDR* gene encodes the vitamin D receptor, which allows the body to metabolize vitamin D [21].
ESR1	Encoding estrogen receptor, believed to have a role in modulating osteoclast differentiation and function. Several polymorphisms in this gene have been connected with the rate of bone loss after menopause[20].

Table 1 Continued

TNF-α	TNF-alpha is believed to reduce osteoblast mediated mineralization while simultaneously inducing osteoclast differentiation [32,5].
RUNX2	Key transcription factor that induces proliferations and differentiation into preosteoblasts and mature osteoblasts and therefore bone formation[2].
SOST	The *SOST* gene encodes the protein sclerostin in osteocytes. The main role of this protein is to inhibit bone formation by interfering with Wnt signaling. Sclerostin may also promote apoptosis in bone cells, further inhibiting bone growth.[21].
SOX9	A critical transcription factor for BMP1 induced chondrocyte differentiation and osteoblast activity[33].
SMAD1	This gene is believed to play a key role in bone development and postnatal bone formation. SMAD proteins control the expression of RUNX2[34].

1.1.5.2 Previous Published GWAS of Bone Health

Genome-wide association studies (GWAS) are a technique for researchers to detect genes involved with human disease. This method searches the genome for small variation, or single nucleotide polymorphisms (SNPs) that are associated with a particular disease or trait. This research allows hypotheses to be made on the gene that influence a person's risk of developing that disease[35]. Previous GWAS have been performed on BMD and bone geometry in order to identify SNPs associated with bone phenotypes as well as to identify new genes that may contribute to the many pathways that control bone homeostasis. Published GWAS have already established 63 genomic loci associated with BMD in adult populations[36]. While individual variants at these loci have modest contributions to the variability in bone health, collectively many variants can significantly account for trait variation between individuals. In performing a GWAS on a common, multifactorial disease, we would expect to find many common genetic variants that on their own have a small impact but together represent a greater cause of variability[26,27,30,37–42].

1.1.5.3 Polymorphisms Known to Influence BMD

Previous GWAS of bone markers have identified several polymorphisms that have associated with BMD and bone health. The majority of these studies have studied adult bone health, however there are several publications that focused on a pediatric population. For example, common variants in the *LRP5* gene are related to BMD and osteoporotic fracture, the *sp1* binding site polymorphism in the *COL1A1* gene has been determined to decrease bone mass and is associated with an increased risk of osteoporotic fractures in elderly women[43], the *PvuII* polymorphism in the *COL1A2* gene is a risk factor for osteoporosis, and the the *PvuII* polymorphism in the *ESR1* gene is associated with BMD in postmenopausal women.

While the vast majority of studies assessing the genetics of bone health have used adult populations, several studies have recently been published investigating the number of BMD-lowering polymorphisms at known adult GWAS-implicated loci to determine an overall genetic risk score for osteoporosis for pediatric patients. A higher genetic risk score had a negative effect on BMD and BMC at the age of 13 and was associated with a slower rate of bone accrual between the ages of 13 and 17[22]. Researchers are beginning to look into the difference in the genetic regulation of bones at difference life stages and finding that bone development during youth is strongly impacted[36].

In the few GWAS, previous to this study, done on childhood bone health, researchers found that there were common genetic influences on BMD in children and in adults, especially in the *ESR1* and Wnt signaling pathway genes[22,44]. For example, Estrogen receptor gene polymorphisms were associated with BMD in adolescent boys[45], *Osterix* and *WNT16*[37] gene polymorphisms were associated with childhood total BMD through a primary effect on growth[44], RANKL polymorphisms were associated with cortical BMD[26], and *VDR* gene polymorphisms were

determined to be associated with child spin BMD[8,46]. A meta-analysis GWAS for BMD as part of the Bone Mineral Density in Childhood Study, researching of longitudinal pediatric BMD measurements, found that several adult BMD associated genes to be associated. This study also found significant associations for SNP-by-age interactions, suggesting multiple variants associated with adult BMD are in fact exerting effects early in life[18]. Lastly, a previous study utilizing the Iowa Bone Development Study investigated the impacted associations of eight candidate genes (*COL1A1/2, osteocalcin, osteonectin, osteopontin, VDR, ERα,* and *AR)* with BMD and BMC and ages 4.5- 6.5 years. This study detected the strongest association with *COL1A2* and *Osteocalcin.* These results also showed a significant gene by gene interaction, suggesting that the combination of genotypes at several loci may be as significant as a single genotype for BMD and BMC[47]. Many of the previous genetic studies on BMD and bone markers during childhood have reported links with variants in osteoblast – related genes (such as *COL1A1/2, osteocalcin, PTHR1, LRP5, and ESR1)*[44]. This is consistent with the idea that bone formation is predominant in childhood, before peak bone mass is reached, and under tight genetic regulation early in life.

1.1.5.4 Genetic Syndromes Associated with Low BMD

Monogenic syndromes associated with low BMD, while not likely the cause of individual variability in bone phenotypes, do pinpoint several genes influencing bone development in which common polymorphisms may influence normal variation in BMD. Several well-known genetic syndromes that are associated with findings of low BMD and early onset osteoporosis include the conditions in Table 2[8,48,49].

Table 2: Genetic Syndromes Associated with Low BMD

Genetic Disorder	Gene	Inheritance
Glycogen Storage Disease 1	*G6PC*	AR
Loeys- Dietz Syndrome Type 2	*TGFBR2*	AD
Osteogenesis Imperfecta	*COL1A1* *COL1A2* *CRTAP* *P3H1*	AD
Hemochromatosis Type 1	*HFE*	AR
Cystic Fibrosis	*CFTR*	AR
Duchenne Muscular Dystrophy	*DMD*	X-linked
Down Syndrome	Chr. 21	Sporadic
Fragile X Premutation Carriers	*FMR1*	X-linked
Beta- Thalassemia	*HBB*	AR
Prader- Willi Syndrome	Chr. 15q11.2-q13	Sporadic
Turner Syndrome	45, X	Sporadic

1.1.5.5 Agonistic Pleiotropy

The differences in genetic regulation and contributors of bone homeostasis at different periods of life can be connected to hypotheses of genetics and aging. George Williams initially proposed the agonistic pleiotropy hypothesis as an evolutionary explanation of biological aging in 1957. Agonistic pleiotropy means that a single gene may influence several phenotypes (pleiotropy) and that these effects may impact fitness in different ways at different life stages. Williams proposed that a gene caused both increased reproduction and fitness in early life and aging in later life. This

idea is built on the idea that organisms are under competing environmental constraints and that genetic variants can be adaptive for growth early in life and maladaptive later in life[50,51]. This hypothesis connects with the previous findings and hypotheses of the genetics of bone development in that as environmental factors and exposures change throughout life, genetic variants can have different impacts and power in bone development at different periods of life.

1.1.6 Environmental Factors and Regulators of Bone Health

While bone health is believed to be highly familial and genetic, it is also influenced greatly by environmental and medical factors[16,27]. Nutrition, physical activity, and hormonal balances represent environmental factors that impact bone development and health and can be targeted for bone health interventions. The expected growths in bone size and mass during childhood and adolescents are only reached when there are favorable environmental factors[19]. Demonstrating this, studies have shown a 35-65% increase in common childhood fractures over the past four decades. These trends raise worries that current lifestyles are compromising childhood bone health. Trends of childhood obesity and a higher body mass index coupled with less nutritious diets and less daily physical activity have a negative impact on early bone health and as an effect, osteoporosis risk later in life[19]

1.1.6.1 Nutrients

Nutrition and bone health are closely connected. A healthy diet can help avoid and or treat bone disease and related musculoskeletal disorders by promoting bone formation and maintenance. Conversely, deficiencies in essential vitamins and minerals can increase risk for poor bone health. Two crucial nutrients for bone health are calcium and vitamin D[52].

Calcium

Calcium is vital for normal skeletal growth and development. Calcium is a major component of bone tissue as the skeleton houses 99% of the body's calcium source[53]. When the body is deficient in calcium, the skeleton acts as a calcium reservoir to tap into. Therefore, when calcium is needed, bone resorption, through osteoclast activity, will prevail to release calcium into the bloodstream. Prolonged and excess osteoclast activity due to low levels of calcium will result in decreased bone mass and low BMD[2]. Adequate calcium intake is crucial for reaching the optimal peak bone mass and lowering the rate of age- related bone loss[52]. A high intake of milk and dairy during childhood is linked with a higher bone mass at maturity[16]. In a study of 10 year old identical twins, calcium citrate supplements given for 3 years significantly increased bone mass when compared to the control group[16]. Additionally, studies have estimated that BMD in the third decade could be increased by 1% with a calcium intake of 200mg per day up to 16% with an intake of 2100 mg per day[19]. Lactose intolerance has also been shown to be connected with lower bone mass and increased bone fragility due to the limited calcium intake[54].

Besides the dietary intake of calcium, the absorption and metabolism of calcium intake is an essential factor in determining the availability of calcium for bone development. Calcium metabolism and the regulation of calcium homeostasis have been shown to have genetic contributors. The literature reports a heritability of calcium metabolism up to 52%[55]. Additionally, studies have begun to see a connection between epigenetics, the study of alterations in gene expression other than sequence changes, and bone development. Researchers have hypothesized that placental calcium transporters may be significant in epigenetic regulation of bone growth[56].

Vitamin D

Vitamin D is a critical factor necessary for mineralization of bone tissue[16]. Vitamin D, a secosteroid, made in the skin through exposure to sunlight and obtained from diet, is a key regulator of calcium and phosphate levels[55]. Vitamin D is an important factor of the strength and mineralization of bone. An insufficient level will induce amplified bone resorption, lead to bone loss, and reduce muscle mass. Muscle loss can impact neuromuscular function and balance; therefore increasing the risk for falls and fractures, further influencing bone health[4]. However, sunlight exposure can result in greater BMD in vitamin D deficient bones and lead to the prevention of further fractures[54]. As of 2010, five out of nine studies researching the impact of vitamin D supplementation alone, and 16 out of 22 studies investigating vitamin D in conjunction with calcium, demonstrated significant positive effects on BMD. Several studies reported significant benefits seen within five weeks of supplementations in participants with vitamin D deficiency and osteoporosis/ osteopenia[57].

Vitamin D metabolism has also been to seen to have genetic regulators, with a heritability ranging from 43%- 65%[55]. Researchers have also implicated an epigenetic effect of maternal vitamin D status during pregnancy. While studies investigating the effect of epigenetics and variability in bone development are in the early stages, these results further demonstrate the vitality of vitamin D in bone development[56].

1.1.6.2 Physical Activity

Weight bearing physical activity causes new bone formation and results in greater bone strength. Strains on bone greater than minimally required for constant remodeling yield a response that stimulates bone production to meet the increasing load requirement. This adaptive response occurs predominantly during times of drastic growth and with habitual physical activity[58]. A previous

study of bone markers and exercise in children of the Iowa Bone Development Study demonstrated that there are statistically significant associations between exercise and bone health during early childhood, well before peak bone mass is reached[58]. Daily exercise alone explains 1.5 to 9% of variance in child bone markers and is associated with bone health throughout childhood[58]. Further, physical activity during childhood and adolescence may in fact account for up to 17% of variance in BMD in the late 20's[16].

Interventions that promote physical activity are needed because young children are not participating in enough physical activity to boost bone health during the period in life where they may be the most active. Studies have demonstrated that physical activity is associated with BMC and bone geometry in a dose dependent manner, and that bone health is significantly improved with high physical activity when compared to physically inactive or moderately active counterparts[59]. Studies have also demonstrated site-specific differences in how the skeleton responds to repetitive physical activity and weight bearing exercises in children. This is especially seen in children involved in sports such as gymnastics and baseball[60]. The influence of sports and physical activity on bone development and mineralization is greatest before puberty compared to exercise in adulthood. Additionally increased bone mass acquired with intensive physical activity in childhood continues into adult life. Former runners, gymnasts, and dancers have BMD measurements as high as 8-12% greater than their age-matched controls, even years after they have retired from the sport.[19]

1.1.6.3 Hormones

Many hormones affect bone growth and remodeling. These include growth hormones, parathyroid hormones, estrogens, calcitonin and thyroid stimulation hormones, osteoprotegrin, leptin, and

serotonin. Parathyroid hormones and sex steroids represent major influencers of bone development and maturation[55].

Parathyroid Hormones

Bone resorption can be triggered by parathyroid hormone (PTH) in response to hypocalcemia. PTH stimulates osteoclast production. PTH has an significant function in bone synthesis and preventing osteoblast and osteocyte apoptosis[61]. Recurrent low level doses of PTH increases osteoblast activity, bone production, and bone mass and is currently an established anabolic treatment for osteoporosis[61]. Heritability of parathyroid hormone regulation is suggested to be 60%.[55]

Sex Steroids

Before puberty is reached, bone growth is largely contingent on growth hormones, however, sex steroids are critical for complete bone maturation and mineral accrual in the teenage years[8]. It is well established that estrogen is the vital regulator of bone metabolism in both men and women, affecting bone growth, remodeling, and homeostasis[8,62]. With roles in the regulation of osteoblast mediated bone synthesis and osteoclast activity in multiple pathways, including progenitor cell recruitment, proliferation, differentiation and apoptosis[8]. One of the best predictors of bone loss in women is estrogen deficiency at the time of menopause. There is a 10-year cumulative loss in BMD of 9-10% associated with menopause[62]. Menopause is the most common cause of osteoclast activity that leads to higher fracture risk and increased mortality of osteoporosis[2].

Androgens also impact bone growth and may contribute to some gender- related differences in bone development. Androgens stimulate male sexual differentiation before birth and sexual maturity during puberty and may provide protection against osteoporosis. During puberty, males develop greater bone mass than females due to increased perosteal apposition. Estrogens in

females also induce epiphyseal closure earlier in women, resulting in longer bones in males. While the rate of osteoclastic activity is similar in both sexes after puberty, the net loss in bone tissue is less in men due to a greater bone length, larger bone perimeters, and a larger cortical volume in males compared to females. Therefore, elderly men maintain cancellous bone integrity and bone strength significantly in comparison with postmenopausal women[63].

1.1.7 Osteoporosis

Known candidate genes of bone biology do not only influence the risk of low BMD. These genes can affect bone growth and development, and can cause diseases that are inherited in a classical Mendelian manner. These disorders include bone dysplasia, osteopetrosis, osteogenesis imperfect, and osteoporotic-psuedoglioma syndrome[13]. While there are a variety of bone and skeletal disorders, osteoporosis is the most common disorder with the largest public health burden. Osteoporosis is a skeletal disorder diagnosed by low bone mass and microarchitectual deterioration of bone tissue, which in turn causes increased bone fragility and susceptibility to fracture. Osteoporosis is diagnosed when BMD lies than 2.5 standard deviations below the average[4].

1.1.7.1 Osteoporosis and Public Health Burden

Osteoporosis risk increases with age and is a major health threat to millions of elderly worldwide[41]. Osteoporosis is a common condition that affects 10.3% of the general population, affecting up to 30% of women and 12% of men at some point in life[48,13]. In the U.S. alone, almost 54 million women and men are affected by osteoporosis and low bone mass[38]. Worldwide, osteoporosis causes more than 8.9 million fractures each year, meaning there is an osteoporotic fracture every three seconds[54].

Osteoporosis is a major cause of morbidity and mortality. Hip fractures, in particular are a common serious concern for individuals with osteoporosis with a high one-year mortality and morbidity rate. Thirty percent of individuals that suffer from a hip fracture die within the first year following the fracture, and many more will experience significant functional loss[64,65]. Though osteoporosis is not the acute cause of death, an osteoporosis related fracture serves as the precipitating event that leads to loss of mobility and subsequent rapid decline in health across months.

The cost of osteoporosis-related treatment in the U.S. was reported as $22 billion in 2008[66]. However, costs of osteoporosis far exceed that of just treatment. Quality of life and ability to work are also strongly impacted. Those diagnosed with osteoporosis are more likely to have a disability (52.6%) compared to unaffected individuals. People affected with osteoporosis also report twice as many mentally and physically poor days as unaffected individuals. Adults with osteoporosis are also more likely to perceive their health as being poor (45.4% reporting health as fair or poor) than individuals with healthy bones[66]. In women over the age of 45, osteoporosis accounts for more hospitalized days than any other disease, including heart disease, diabetes, and breast cancer[54].

With the trend of an aging population, the public health threat of osteoporosis continues to affect more patients with an increasing cost of treatment. It is predicted that if these trends continue, 61 million individuals in the U.S. will be affected with osteoporosis by the year 2020[54]. Even if the rates of osteoporosis among the elderly remain constant, the aging trend of the world population is believed to increase the number of osteoporotic bone fractures and treatment costs by 48%, resulting in more than 3 million fractures and treatment costs of $25.3 billion by 2025 in the U.S[67].

1.1.7.2 Risk Factors for Osteoporosis

Many contributors impact osteoporosis lifetime risk. These factors include diet, physical activity, mediation use, and coexisting conditions. The first major risk factor is age. The age related increase in oxidative stress has the most impactful detrimental effect on bone homeostasis. Men and women both experience a progressive decline in BMD with age that begins as soon as peak bone mass is reached[4]. In the elderly, the strongest clinical risk factor is a positive family history for osteoporosis, emphasizing the genetic implications of bone health[13].

Race has been shown to impact BMD. BMD has been shown to be significantly higher in individuals of African descent and osteoporosis incidence is higher among Caucasian and Asian individuals[66,68].

A poor diet plays a significant role in the risk of osteoporosis. The dietary intake of the essential vitamins, such as vitamin D and calcium, explained previously, a higher protein intake is also associated with a lower rate of age-related bone loss. Additionally, high intake of alcohol also results in a significant risk of fractures due to reduced BMD and BMC[54]. Chronic excessive drinking affects bone metabolism and inhibits bone formation while increasing bone resorption[4].

Smoking can also lead to reduced BMD and increased bone fragility. The mechanism in which smoking results in poor bone health is not yet clear because smoking negatively impacts many body tissues and regulatory pathways[4].

Physical inactivity and a sedentary lifestyle are major risk factors for developing osteoporotic fractures. Impaired neuromuscular function, including weakened muscle strength and impaired gait and balance, are also risk factors for fractures. Studies have repeatedly demonstrated that BMD in postmenopausal women can be preserved or increased with therapeutic exercises.

However, low body weight and extreme weight loss is related with increased bone loss and a higher risk of fracture[69].

Several medical conditions and medications can cause secondary osteoporosis. Conditions known to cause secondary osteoporosis include rheumatoid arthritis, hypogonadism, hyperparathyroidism, hyperthyroidism, kidney disease, GI disorders, such as Crohn's and celiac disease, and Type 1 Diabetes[69]. The additional consequence of prolonged use of medications such as corticosteroids, anxiolytics, proton pump inhibiting drugs, neuroleptics, sedatives, and antidepressants have also been shown to significantly increase the risk of osteoporosis-related fractures[48].

An advantage of pediatric cohorts is that while osteoporosis risk is the interaction of genetic factors and environmental risk factors such as physical activity, alcohol, smoking, and medications, children because have limited exposures (in amount and time period exposed) to the environmental risks compared to adults. Because of this, the genetic assessment and genes found to be associated with pediatric bone health may have more power due fewer environmental and behaviors confounding variables.

1.1.7.3 Current Interventions for Osteoporosis

Treatment of diagnosed osteoporosis is cost-effective no matter the age in which treatment is begun[54]. Recognizing and treating patients at risk for fracture before the first osteoporotic related fracture will substantially lessen the long-term burden of the disease. Reducing the chance of the first fracture from 8% to 2% can reduce the 5-year fracture risk from approximately 34% to 10%[54]. However, poor compliance is the most significant issue with osteoporotic treatment. Studies have shown that only 40% of patients still continue treatment beyond a year and that only 20% of patients are compliant after two years[54].

As the biology of bone disease is better understood, better treatments and therapies for osteoporosis are available. Treatments depend on the current knowledge of osteoblast and osteoclast pathways in order to either inhibit osteoclast activity or increase osteoblast activity. Several current approved therapies include cytokine inhibition, inhibition of osteoclastogenesis pathways, blocking osteoclast activity, enhancing osteoblast activity, and interfering with osteoblast inhibition. Several treatment strategies of osteoporosis have utilized known candidate genes of bone growth. One example includes, Denosumab, an anti-osteoclast treatment that targets RANKL with a human monoclonal antibody. Osteoblast differentiation is also promoted by a pro-anabolic treatment that targets LRP5 by inhibiting DKK-1 activity. Another example is the inhibition of sclerostin to interfere with osteoblast inhibition due to studies that demonstrated *SOST* gene (which encodes sclerostin) mutations were associated with high bone mass[2].

BMD screening has become widely recommended. The U.S. Prevention Services Task Force has recommended that all women 65 years or older without fractures or secondary osteoporosis and women less than 65 years with a 10 year fracture risk greater or equal to that of a 65 year old women should have BMD screening through DXA[66]. Additionally patients and providers can assess the 10-year fracture risk with the FRAX risk assessment tool available through the World Health Organization[66]. Because osteoporosis and related costs of mortality and morbidity pose such a large public health burden, implementation of effective treatments and prevention protocols are crucial in the hope of promoting healthier bones on a population scale.

1.2 PUBLIC HEALTH SIGNIFICANCE OF GWAS OF CHILDHOOD BONE PHENOTYPES

Skeletal growth and bone health during childhood and adolescence is a strong predictor of the lifetime risk of osteoporosis, which poses a serious public health threat worldwide. Due to the significant influence of behavioral factors early in life, tight genetic regulation of bone homeostasis during early bone development, and the fact that the majority of bone formation is reached during adolescence, osteoporosis should be considered a delayed onset disease with pediatric origins. Despite advances in treating bone fragility, there is currently no cure for osteoporosis. More research is needed to fully understand the causes of individual variation in bone health and early prevention strategies to lower osteoporosis risk[8].

With the ultimate goal of preventing osteoporosis, it is important we increase our knowledge of the pathologic mechanism of the disease. While the knowledge of genetic factors influencing bone health and osteoporosis have increased dramatically, there are still many unanswered questions. First, the bulk of genetic association studies have been performed in with adult populations and adult bone measurements, though the genetic regulation of bone health is possibly stronger and more impactful during early development. Secondly, we do not currently understand gene-gene interactions in bone-related pathways or the effect of interactions between genetic variations and environmental factors.

This study is one of the first GWAS investigating bone health in children. This is also a wide-ranging GWAS in which we investigated associations with ten different bone health phenotypes across multiple skeletal sites. The public health significance of this study is that if we know the genetic contributors and polymorphisms associated with poor bone health and a genetic predisposition to osteoporosis later in life, then we can detect these genetic variants in order to

identify children with greater genetic risk. This will allow for more targeted interventions in those most at risk to hopefully lower the incidence of osteoporosis in the future.

New approaches towards treatment and, more importantly, osteoporosis prevention will require efforts in which the progress and insights from biological research are paired with clinical utility[20]. Because the foundation of bone health is established so early in life, osteoporosis prevention interventions should begin by heightening bone health throughout childhood[19]. This research can reach clinical and public health practices if one day genetic screening programs are available to identify children with a genetic predisposition to osteoporosis later in life. Effective bone health interventions, including promoting healthy nutrition and exercise, can then be recommended and initiated early in "high-risk" children, optimizing peak bone mass reached.

1.3 RESEARCH QUESTIONS AND SPECIFIC AIMS

1.3.1 Research Questions

Question 1:

Are there loci that are significantly associated with childhood bone health phenotypes?

Question 2:

Do significant loci contain genes with function related to bone development and homeostasis?

1.3.2 Specific Aims

Aim 1:

Perform a genome-wide association analysis for ten bone health phenotype measurements obtained on 296 Caucasian children (average age 5y) from the Iowa Bone Development Study in order to identify SNPs associated with bone health early in life.

Aim 2:

Perform annotation of the neighboring genes of significant and suggestive SNPs (p-values less than 10^{-6}) associated with each bone phenotype. Develop a working mechanism or pathway, based on previous literature, of the genetic link to bone health.

2.0 METHODS

2.1 STUDY POPULATION

The Iowa Bone Development Study was initiated in 1998 and is a longitudinal study of childhood, adolescent, and young adulthood bone health. Children and parents for this study are volunteer participants from 890 families previously recruited to participate in the Iowa Fluoride Study[70]. Infant participants and their parents were originally recruited from eight Iowa hospitals between 1992 and 1995 immediately postpartum and followed-up throughout infancy, childhood, and adolescence[58]. The ongoing study is assessing bone development over time through assessments including bone mineral composition and geometry, dietary intake, physical activity, anthropometric measurements, demographics, and parental factors[71]. Assessments of bone development were made at target ages 5, 9, 11, 13, 15, and 17 years. The study is currently completing bone assessments of participating young adults who are now ages 19-23 years[72]. This study will focus on assessments when the participants were ages four to seven years old (mean age = 5.3 years). This cohort includes 296 healthy, Caucasian children that completed bone measurements. Written informed consent was provided by the parents and assent was provided by child participants. The study was approved by the University of Iowa Institutional Review Board (Human Subjects)[71]. The analysis of this data was approved by the University of Pittsburgh Institutional Review Board.

2.2 BONE PHENOTYPES

Bone mineral density (BMD) and bone mineral content (BMC) measurements were derived from whole body and left hip scans using a Hologic 2000 dual-energy x-ray absorptiometer (DXA) with a fan-beam geometry and a multiple detector array[58]. To reduce operator-related variability, all measurements were performed on the same device and quality control scans were conducted daily[11]. Coefficients of variation were found to be 1-2% in BMD of the hip, spine, and total body[58]. Studies have consistently demonstrated DXA to be an accurate and precise tool to assess lean tissue mass when animal subjects, approximately the weight of a child, were studied[73]. Further, DXA is the preferred method for evaluating child BMD because it is believed to be safe, precise, low in cost, and associated with a low dose of radiation[15,74–76].

Bone mineral density (in units of grams/ square centimeters) is the relative value of amount of bone mineral divided by the 2-dimensional projected area of the selected bone. Bone mineral content (in units of grams) reflects the absolute amount of mineral in the selected bone[58]. The following mineral measurements were obtained: hip, spine, and head bone mineral density (g/cm^2) and hip, spine, head, and whole body (excluding head) bone mineral content (g).

Structural bone geometry measurements were determined from hip DXA images using the Hip Structure Analysis program (HAS version 2.1). The program locates the cross-section traversing the femoral neck at its narrowest point and the following measurements are obtained: femoral neck cross-sectional area (CSA, cm^2), femoral neck width (cm), and femoral neck section modulus (cm^3)[11]. While lower BMD is a strong determinant of bone strength, bone geometry is also a major determinant of osteoporosis risk. Little work has been done to determine genetic regulators of bone geometry; these measurements may in fact be better predictors of fracture risk and monitoring responses to osteoporosis therapy. This study is one of the first GWAS studying

bone geometry measurements in children. More extensive details regarding the measurements this program uses to calculate structural geometry have previously been extensively described[11].

2.3 COVARIATES

Data on several potential covariates were collected, and included in the tests of genetic association: sex, height (cm), weight (kg), age (years) at the time of the bone measurements. The rationale for making covariate adjustments was that these factors may be sources of considerable phenotypic variance that may obscure the comparative smaller portions of variance attributable to specific genetic variants. Therefore, making covariate adjustments may aid in identifying the associated genetic variants. Furthermore, some of the covariates (e.g., height and weight) are themselves under genetic control, but identifying the genes influencing these is not desirable for the goals of this particular study. By adjusting for these covariates, our genome-wide scans will be insulated from the genetic factors influencing body size, rather than bone, per se. In other words, covariate adjustment helps ensure that associated genetic variants will be reflective of specific mechanisms relevant to bone phenotypes, rather than reflective of mechanisms relevant to general growth and body size.

2.4 GENOTYPING AND IMPUTATION

Details regarding allele calling, data cleaning, and quality assurance metrics have been previously described in detail[77] and are also publicly available from dbGAP[78,79].

In brief, the Illumina Human610-Quadv1_B BeadChip[80] and Illumina Infinium II assay protocol[81] were used for this study. All genotyping was performed on behalf of the NIH GENEVA consortium by the Johns Hopkins University Center for Inherited Disease Research (CIDR). Among 620,901 SNPs released by the CIDR, 2,671 SNPs were filtered out due to the Hardy-Weinberg Equilibrium test filter P-value less than 0.001; 32,417 SNPs were excluded from analysis due to a missing call rate greater than 10%; 69,818 SNPs were filtered out due to a minor allele frequency less than 2%. After applying these filters, 548,051 SNPs were used in the statistical analysis. 296 children were successfully genotyped[82].

Imputation was performed in order to produce information on unobserved SNPs as well as fill in sporadic missing genotype calls among observed SNPs. This was accomplished using publically available haplotype data from the 1000 Genomes Project Phase 3 reference panel[83]. Pre-phasing was performed using SHAPEIT2[84] and imputation was performed using IMPUTE2[85,86]. In total, 7.4 million[87] (https://mathgen.stats.ox.ac.uk/impute/1000GP_Phase3.html) SNPs passing the Hardy-Weinberg Equilibrium and minor allele frequency filter were imputed.

2.5 PRINCIPAL COMPONENTS OF ANCESTRY

A limitation of GWAS is that population stratification can be a confounding variable. If not accounted for, population stratification can yield false positive associations[88,89]. Principal component analysis is a statistical technique that can be used to identify structure in the distribution of genetic variation across ethnic backgrounds and to adjust for ancestry[90]. Including principal

components of ancestry in tests of genetic associations avoids false positive findings due to epidemiological confounding by ancestry-informative genetic variants.

The principal components were generated using PLINK (version 1.9)[91,92]. To develop a set of SNPs on which to perform PCA, SNPs with a minor allele frequency less than 0.05 and SNPs missing for more than 5% of the participants were removed, yielding 518,187 SNPs. This set of SNPs was then pruned to remove blocks of highly correlated, highly variable SNPs using pairwise correlation within a sliding window of 50 SNPs (as implemented in PLINK[91,92]). After linkage disequilibrium-based pruning, 95,384 SNPs were retained and included in the principal component analysis. Our GWAS analysis included adjustment for one principal component of ancestry which was sufficient to capture population structure because the study population consisted of all Caucasian children from Iowa (i.e., a fairly genetically homogenous population).

2.6 STATISTICAL ANALYSIS AND RESULTS ANNOTATION

Association between each bone health phenotype and each SNP was tested using the genetic software PLINK[91,92]. SNP filters for association scans included:

Table 3: Filters Included for Association

Filter	Effect on Association	Threshold
Missingness Per Individual	Individuals removed for low genotyping	call rate > 0.1
Allele Frequency	SNPs failed frequency test	minor allele frequency < 0.02
Missingness Per Marker	SNPs failed missingness test	call rate > 0.1
Hardy- Weinberg Equilibrium	Markers excluded based on Hardy-Weinberg Equilibrium (HWE) test	$P \leq 0.0001$

Linear regression was used to test each SNP individually under the additive genetic model while simultaneously adjusting for covariates and principal components of ancestry. Results were visualized by creating Manhattan plots, quantile-quantile (Q-Q) plots, and calculating genomic inflation factors (λ) using the RStudio statistical environment[93]. Following, the adopted standard in the field, genome-wide significance was reached if a SNP P-value surpassed the $P = 5 \times 10^{-8}$ threshold, this is an extremely conservative threshold based on a Bonferroni correction for one million SNPs[94]. This threshold is represented by a solid line on each phenotype Manhattan plot. Because we view GWAS as a hypothesis-generating approach, we additionally scrutinized "suggestive" associations that passed the threshold of $P < 10^{-6}$.

LocusZoom[95] was used to plot association signals for loci of interest meeting significant and suggestive P-value thresholds. All genes within the 400kb flanking region of significant SNPs were annotated. Based on gene function (including previous research supporting a role in bone development and bone homeostasis, previously reported genetic associations with BMD or osteoporosis, previous model organism studies, and associated transcription factor binding sites,

and suggested interactions) and proximity to the significant SNP, we selected promising genes to report as possible genetic contributors and susceptibility genes to childhood bone health.

To present an illustration of the detailed literature review performed on implicated genes, pathways were constructed. These pathways show the interactions of the promising genes detected in this study and well-known candidate genes in bone biology. These pathways also demonstrate the possible mechanism in which a variation in the implicated genes could lead to variation in bone health. Pathways based on previous studies were created using the ePath3D Online software[96].

3.0 RESULTS

Sample characteristics are summarized in Table 4. Our study population included 296 Caucasian children with a mean age of 5.3 years. Males accounted for 46.96% of the population and females accounted for 53.04%. The mean and range for each bone health phenotype are included in the table below.

Table 4: Descriptive Statistics of Study Population

Sample Characteristics	
Sample Size	296
Percent Male	46.96 %
Percent Female	53.04 %
Mean Age (Range) (years)	5.282 (4.350 – 7.900)
Mean Height (Range) (cm)	111.8 (98.5 – 135.2)
Mean Weight (Range) (kg)	20.48 (13.30 – 44.80)
Mean Hip BMD (Range) (g/cm^2)	0.5565 (0.4203 – 0.7192)
Mean Hip BMC (Range) (g)	6.015 (3.104 – 13.136)
Mean Spine BMD (Range) (g/cm^2)	0.5324 (0.3646 – 0.6853)
Mean Spine BMC (Range) (g)	14.418 (7.988 – 23.823)
Mean Head BMD (Range) (g/cm^2)	1.375 (1.006 – 1.859)
Mean Head BMC (Range) (g)	244.0 (162.3 – 342.8)
Mean Whole Body (excluding head) BMC (Range) (g)	254.25 (79.42 – 556.75)
Mean Femoral Neck Cross- Sectional Area (Range) (cm^2)	0.9084 (0.5700 – 1.5610)
Mean Femoral Neck Width (Range) (cm)	2.116 (1.635 – 2.728)
Mean Femoral Neck Section Modulus (Range) (cm^3)	0.2833 (0.1530 – 0.5830)

The results of the statistical analysis and gene annotation are organized below by the phenotype of the genome-wide association scan. While the results are reported separately by phenotype, we hypothesize that the genetic associations are not necessarily specific to one skeletal site. Our goal was to determine plausible genes that play a role in the genetic regulation of bone homeostasis of children before peak BMD attainment is reached. While genetic contributions to

bone health can differ by skeletal site, our goal for this study was primarily understanding, in general, the possible roles and interactions the following genes play in the intricate processes of bone development and remodeling during childhood years.

GWAS of ten phenotypes were performed. Phenotypes are grouped in this chapter into the categories BMD, BMC, and bone geometry. For each bone phenotype we present results in the following order: First, the loci reaching genome-wide significance are outlined with a focus on the most plausible genes based on known function relevant to bone regulation. A complete list of all the genes surrounding each significant SNP can be found in Appendix A. Following this, the loci reaching suggestive significance are enumerated. Again, genes that were found to have a connection with bone biology are emphasized. A complete list of all of the genes in the vicinity of suggestive loci along with the LocusZoom plots of all suggestive loci in Appendix B.

Again, the nominated genes of this study are simply the candidate genes with the clearest biologically plausible story. We cannot determine from the statistical evidence if genetic variation influencing these genes is truly involved in normal variation of bone development.

Q-Q plots were created for each phenotype to determine the genomic inflation factor (λ). An inflation factor of 1.0 indicates that there is no evidence of systemic p-value inflation due to inadequate adjustment for population structure or model misspecification. The lambda values for the bone phenotypes range from 0.999135 to 1.012036, suggesting that there was no significant systemic bias. The Q-Q plots for each phenotype can be found in Appendix C.

Table 5: Genomic Inflation Factor Values for Bone Phenotypes

Phenotype	Genomic Inflation Factor (λ)	Standard Error
Hip BMD	1.000347	2.182E-6
Hip BMC	0.999135	2.385E-6
Spine BMD	1.007295	1.726E-6
Spine BMC	1.002187	2.126E-6
Head BMD	1.003153	1.914E-6
Head BMC	1.010449	2.473E-6
Whole Body BMC (excluding head)	1.000723	2.331E-6
Femoral Neck Cross- Sectional Area	1.009225	1.949E-6
Femoral Neck Width	1.009364	3.730E-6
Femoral Neck Section Modulus	1.012036	2.958E-6

3.1 BONE MINERAL DENSITY GWAS

The significant and suggestive signals detected through the GWAS of hip, spine, and head BMD are presented in Table 6.

Table 6: GWAS Findings for BMD Phenotypes

Phenotype	Number of Significant Loci	Number of Suggestive Loci	Implicated Genes
Hip BMD	1	3	*COL13A1*
Spine BMD	0	2	----------
Head BMD	0	1	----------

3.1.1 Hip Bone Mineral Density GWAS

The Manhattan plot for the GWAS of hip BMD is shown in Figure 1.

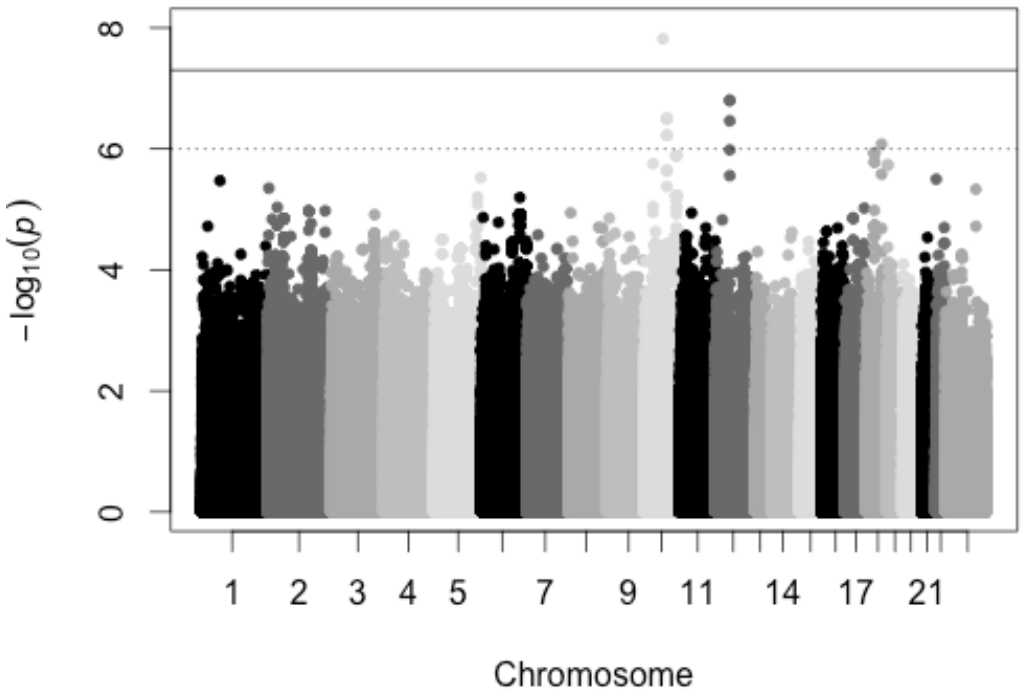

Figure 1: Manhattan Plot for Hip BMD

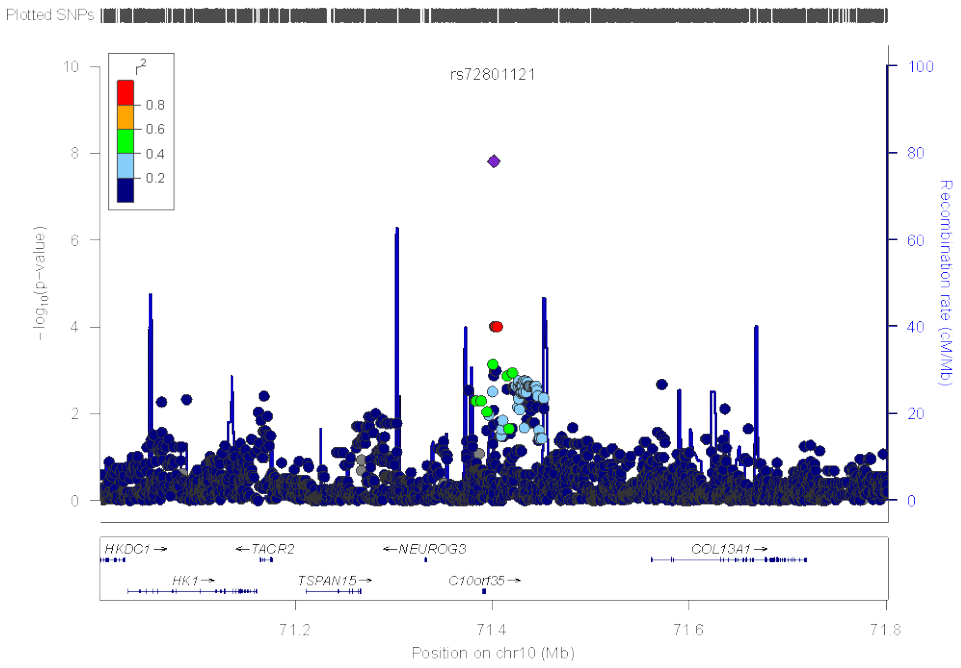

Figure 2: LocusZoom Plot for Significant Loci Associated with Hip BMD

Figure 2 shows the genome-wide significant (P-value = 1.514E-08) association with hip BMD observed for rs72801121 on chromosome 10. Of the genes in this region, the *COL13A1*

gene, immediately downstream of the associated SNP, has the strongest relation to bone biology. *COL13A1* encodes the alpha chain of a nonfibrillar collagen. While the specific function of this protein is not yet known, its expression in all connective tissue producing cells suggests a general role in connective tissues. Unlike the majority of the collagens, which are released into the extracellular matrix, collagen XIII (which is a trimer composed of one or more alpha chain isomers), contains a transmembrane domain, and the protein complex is localized to the plasma membrane[97]. Previous studies have suggested that collagen XIII is involved in the cell-matrix and cell-cell adhesion. Collagen XIII may therefore be involved in endochondral ossification. In mice studies, overexpression of collagen XIII caused a massive bone- overgrowth, however there were no defects in early skeletal development, suggesting that collagen XIII is an important regulator in the early osteoblast differentiation and bone remodeling[98]. COL13A1 is also suggested to have a role in the coupling of bone mass and mechanical stress[99].

Three suggestive signals were observed for the hip BMD phenotype. The table below enumerates the genes surrounding the suggestive loci that were found to have plausible functions related to bone biology and a possible connection to childhood bone health. A suggestive signal on chromosome 12 is especially of interest because the neighboring region includes a homeobox C gene cluster, a highly conserved family believed to have a role in cartilage differentiation and regulation. Additionally several genes in this region have been implicated in a previous GWAS meta-analysis for BMD and risk of fracture in adults[42].

Table 7: Suggestive Genes Associated with Hip BMD

Suggestive SNP	Gene	Chr.	Suggestive P-value	Gene Function Relevance to Bone Biology
rs57205518	*CDHR1*	10q23.1	3.161e-07	This gene is a member of the cadherin family of calcium-dependent cell adhesion molecules and may be related to calcium homeostasis[100].
rs2366139	*ATF7*	12q13.13	1.578e-07	The loci was identified in a GWAS for adult height[101]. The gene also is believed to have a role in vitamin D hypersensitivity of osteoclast precursors in Paget's disease[102].
rs2366139	*HOXC10*	12q13.13	1.578e-07	Another cluster of the highly conserved homeobox genes were identified. Hoxc10 knockout mice have bone changes in thoracic, lumbar, sacral vertebrae, the pelvis, along with changes in the development and ligaments of the hindlimbs. Implicated for a role in the regulation of chrondrogenesies and bone formation in the hindlimb, and a specific role in shaping femoral architecture[103].
rs2366139	*HOXC8*	12q13.13	1.578e-07	This gene product may be involved in the regulation of cartilage differentiation[104]. It could also influence chondrodysplasias or other cartilage disorders[105]. The interaction between this gene and SMAD1 have demonstrated the induction of osteoblast differentiation and bone formation[106].
rs2366139	*HOXC5*	12q13.13	1.578e-07	Identified in a previous GWAS meta-analysis for bone mineral density and risk of fracture[42].
rs2366139	*NFE2*	12q13.13	1.578e-07	Mice deficient in this transcription factor have an increased bone mass phenotype and greater total bone area, cortical bone area and cortical thickness as well as increased BMD[107,108].
rs2366139	*HNRNPA1*	12q13.13	1.578e-07	Mutations in this gene are associated with body myopathy with early-onset Paget disease without frontotemporal dementia type 3. This is an autosomal dominant disease described with

Table 7 Continued

				disabling muscle weakness and osteolytic bone lesions[109].
rs2366139	*ATP5G2*	12q13.13	1.578e-07	Identified in previous GWAS for adult height loci[110].
rs2366139	*CALCOCO1*	12q13.13	1.578e-07	Mouse knockout studies show 85-95% notable differences in BMD and BMC[111].
rs2366139	*HOTAIR*	12q13.13	1.578e-07	Targeted deletion of HOTAIR RNA in mice studies leads to changes in the spine and malformation of bones. This gene may be involved in maintaining the chromatin state of Homeobox genes.[112]
rs2366139	*HOXC11*	12q13.13	1.578e-07	Gene expression is detected in the posterior neural tube, prevertebrae dorsal root ganglia, and hindlimbs. Expression is seen in the mesenchyme posterior to the region forming the femur. Previous studies demonstrate severe malformations of the appendicular skeleton in mice overexpressing hoxc11[113].
rs2366139	*HOXC4*	12q13.13	1.578e-07	Identified in a previous GWAS meta-analysis for BMD and risk of fracture[42].
rs2366139	*HOXC6*	12q13.13	1.578e-07	Identified in a previous GWAS meta-analysis for BMD and risk of fracture. Several targets and pathways regulated by HOXC6 impact bone development and may facilitate metastasis of prostate cancers to the bone microenvironment[114]. BMP7 is a direct repression target of HOXC6 that induces bone formation. While microdeletions involving the HOXC gene cluster are rare, phenotypic features of this deletion include skeletal anomalies, dysmorphism, and intellectual disability[115].
rs9960845	*CCBE1*	18q21.32	8.350e-07	The function of this gene includes calcium ion binding and collagen binding[116].
rs9960845	*PMAIP1*	18q21.32	8.350e-07	PMAIP1 deficient mice exhibited severe osteoporotic phenotype due to an increased level of osteoclasts due to a prolonged survival of osteoclasts[117].

3.1.2 Spine Bone Mineral Density GWAS

The Manhattan plot for the GWAS of spine BMD is shown in Figure 3.

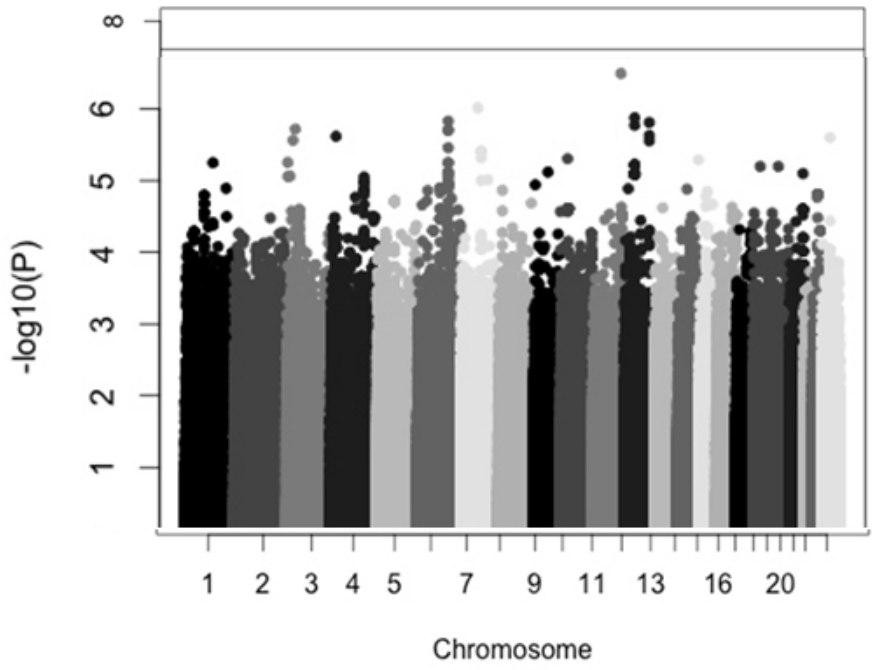

Figure 3: Manhattan Plot for Spine BMD

No genome- wide significant signals were detected for this phenotype, however, two suggestive significance signals were observed for the spine BMD phenotype.

Table 8: Suggestive Genes Associated with Spine BMD

Suggestive SNP	Gene	Chr.	Suggestive P-value	Gene Function Relevance to Bone Biology
7-64494136	*ZNF92*	7q11.21	9.675e-07	The *ZNF92* gene promoter is a transcription factor binding site for the *STAT5B* gene[118]. The *STAT5B* gene may regulate the pattern of long bone growth in males that is found in many species[119].
11-121887592	*UBASH3B*	11q24.1	3.204e-07	The *UBASH3B* gene stimulates negative regulation of bone resorption by osteoclasts[120].
11-121887592	*HSPA8*	11q24.1	3.204e-07	The *HSPA8* gene promoter is a transcription factor binding site for the *STAT1* and *STAT3* genes[121].

3.1.3 Head Bone Mineral Density GWAS

The Manhattan plot for the GWAS of head BMD is shown in Figure 4.

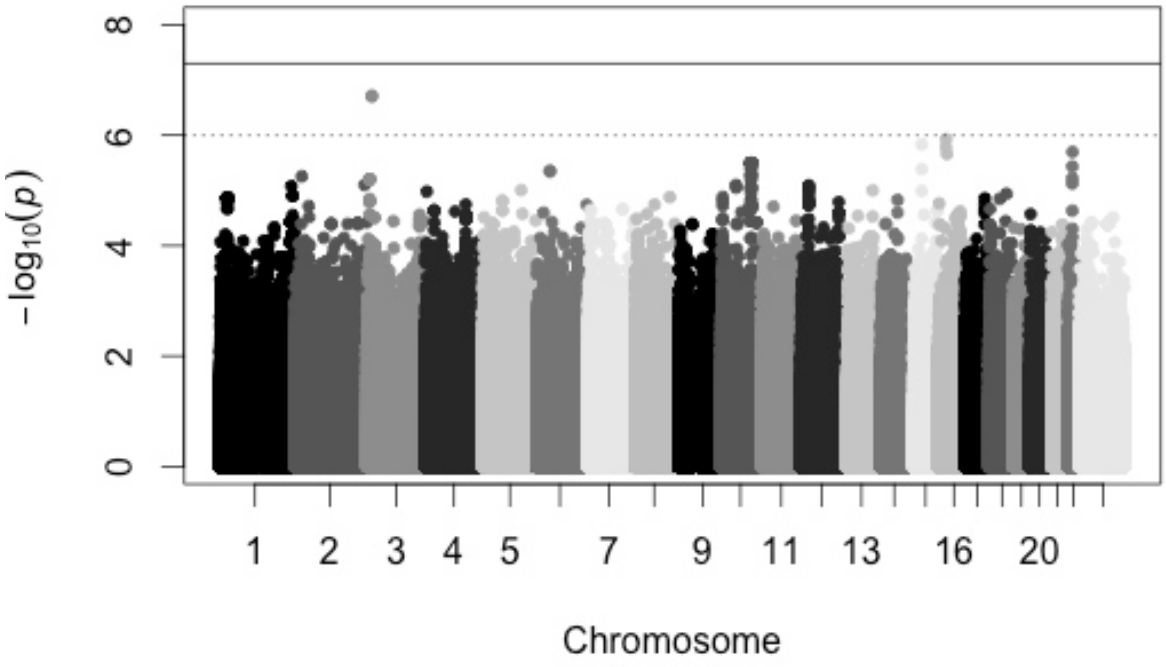

Figure 4: Manhattan Plot for Head BMD

No genome- wide significant signals were detected for this phenotype, however, one suggestive signal was observed for the head BMD phenotype.

Table 9: Suggestive Genes Associated with Head BMD

Suggestive SNP	Gene	Chr.	Suggestive P-value	Gene Function Relevance to Bone Biology
rs76178935	*ANKRD28*	3p25.1	1.952e-07	The ANKRD28 gene is overexpressed in the bone tissue[122].

3.2 BONE MINERAL CONTENT GWAS

The significant and suggestive signals detected through the GWAS of hip, spine, head, and whole body (excluding head) BMC are presented in Table 10.

Table 10: GWAS Findings for BMC Phenotypes

Phenotype	Number of Significant Loci	Number of Suggestive Loci	Implicated Genes
Hip BMC	1	1	*COL11A1*
Spine BMC	0	2	----------
Head BMC	0	3	----------
Whole Body (excluding head) BMC	0	1	----------

3.2.1 Hip Bone Mineral Content GWAS

The Manhattan plot for the GWAS of hip BMC is shown in Figure 5.

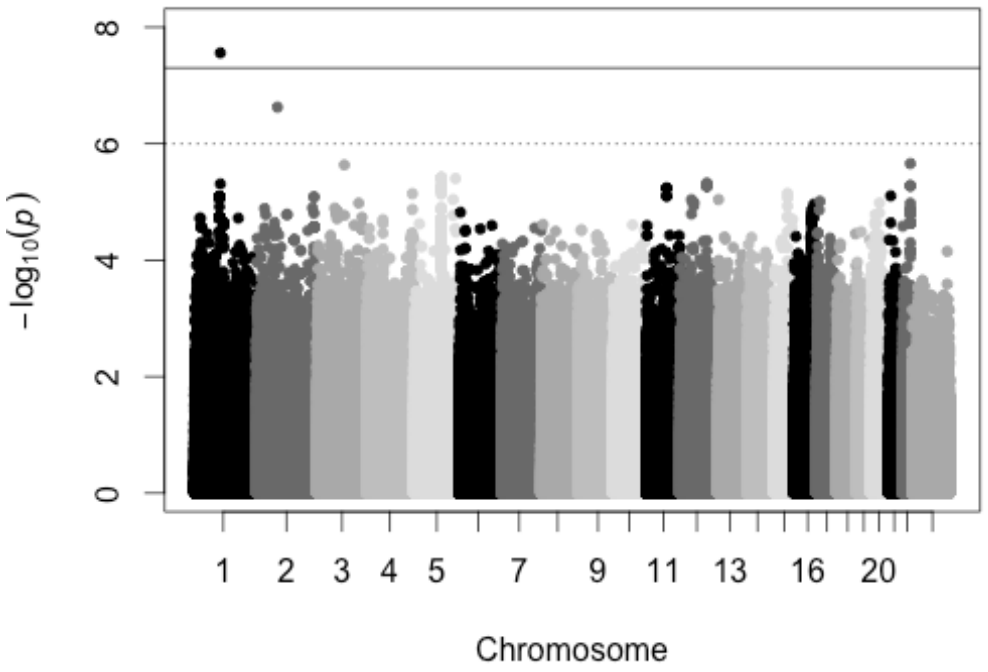

Figure 5: Manhattan Plot for Hip BMC

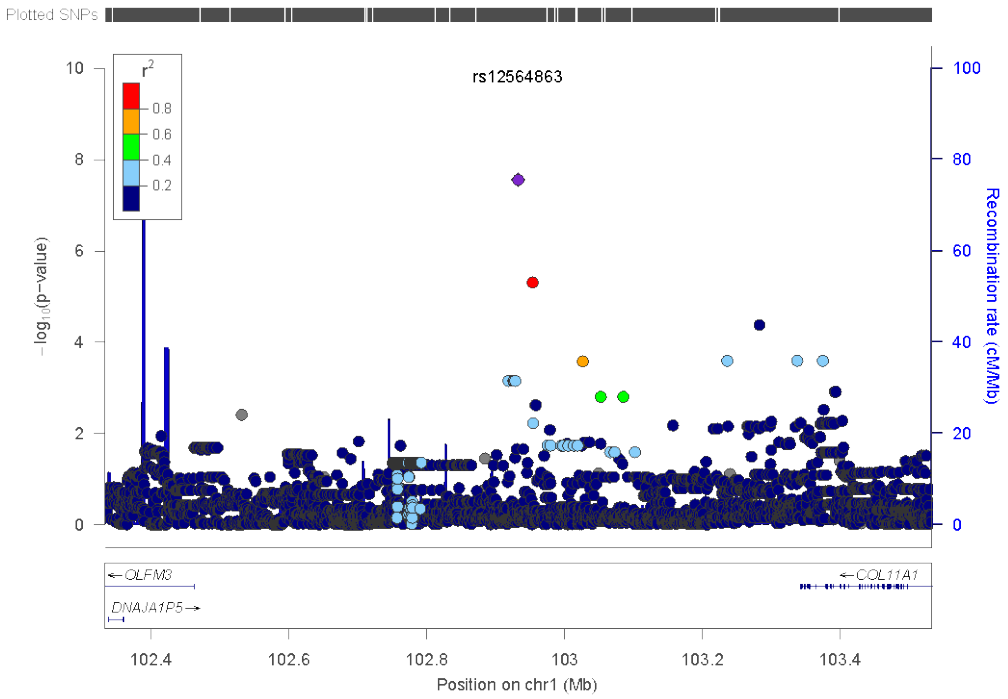

Figure 6: LocusZoom Plot for Significant Loci Associated with Hip BMC

Figure 6 shows the significant (P-value = 2.756e-08) loci with hip BMC observed for rs12564863 on chromosome 1. The significant association was observed downstream of another collagen, *COL11A* which has plausible biological roles related to bone development. This gene encodes one of the two alpha chains of type XI collagen, a minor fibrillar collagen. Collagen is an integral aspect of the extracellular matrix structure, providing strength to the connective tissues that support the body. Type XI collagen is typically found in cartilage, of which the majority is then converted to bone[97]. Mutations in this gene cause Type II Stickler syndrome and Marshall syndrome. Stickler syndrome is associated with hearing and vision loss as well as abnormalities of the bones and joints[123]. A previous GWAS found that polymorphism in this gene is also associated with lumbar disc herniation[124]. This loci was also identified in a GWAS meta-analysis associated with bone mineral density and risk of fracture[42]. A number of previous studies implicate the COL11A1 gene as an essential gene for normal skeletal development. COL11A1 is believed to play an essential role in endochondral ossification, and negatively regulating osteoblast

maturation[123,125]. Additionally, COL11A1- deficient mice were found to have changes in bone microstructure[125].

One suggestive signal was observed for the hip BMC phenotype. The most significant SNP was rs71337766 on chromosome 2 (P-value = 2.357 x 10^{-7}). No genes in this region have functions related to bone biology or a connection to childhood bone health.

3.2.2 Spine Bone Mineral Content GWAS

The Manhattan plot for the GWAS of spine BMC is shown in Figure 7.

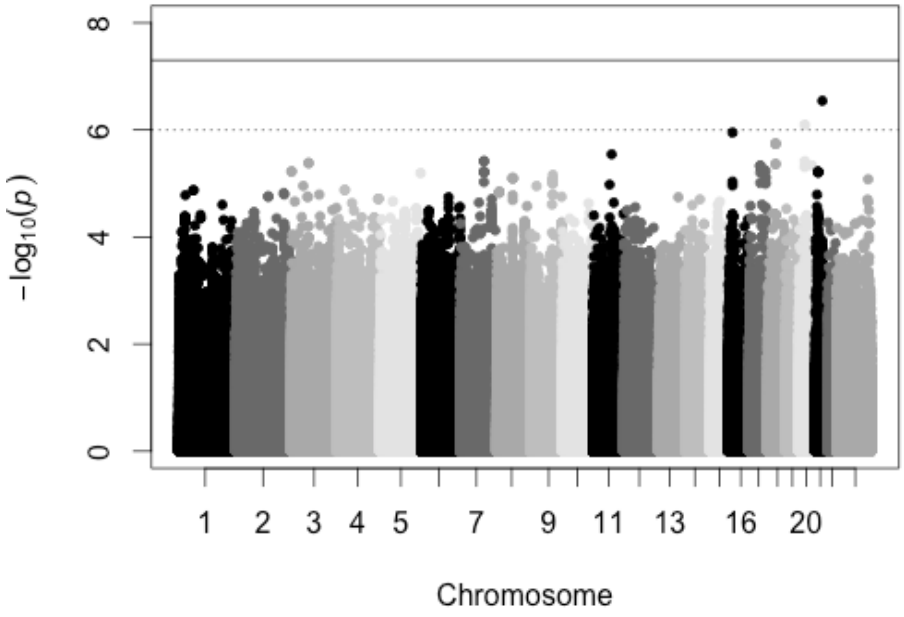

Figure 7: Manhattan Plot for Spine BMC

For the spine BMC phenotype, no genome-wide significant signals were detected. However, two suggestive signals were observed.

Table 11: Suggestive Genes Associated with Spine BMC

Suggestive SNP	Gene	Chr.	Suggestive P-value	Gene Function Relevance to Bone Biology
rs8132933	*DSCR4*	21q22.13	2.850e-07	The *DSCR4* gene is included in the Down syndrome critical region and has been linked to the pathogenesis of Down syndrome. A small number of studies have indicated that bone density in adults with Down syndrome is lower than in the general population and thus increasing the risk for osteoporosis, especially in the spine. Childhood BMD in individuals with Down syndrome was compared to a control group and found to be significantly lower. There was also found to be an approximately two year delay in the BMD reference curve[126]. Additionally this gene promoter is a transcription factor binding site for the *ATF-2*, *PPAR- Gamma-1* genes[127]. *ATF*-2 may be involved in trabecular bone formation and *PPAR- Gamma*-1 promotes osteoclastic activity[128,129].

3.2.3 Head Bone Mineral Content GWAS

The Manhattan plot for the GWAS of head BMC is shown in Figure 8.

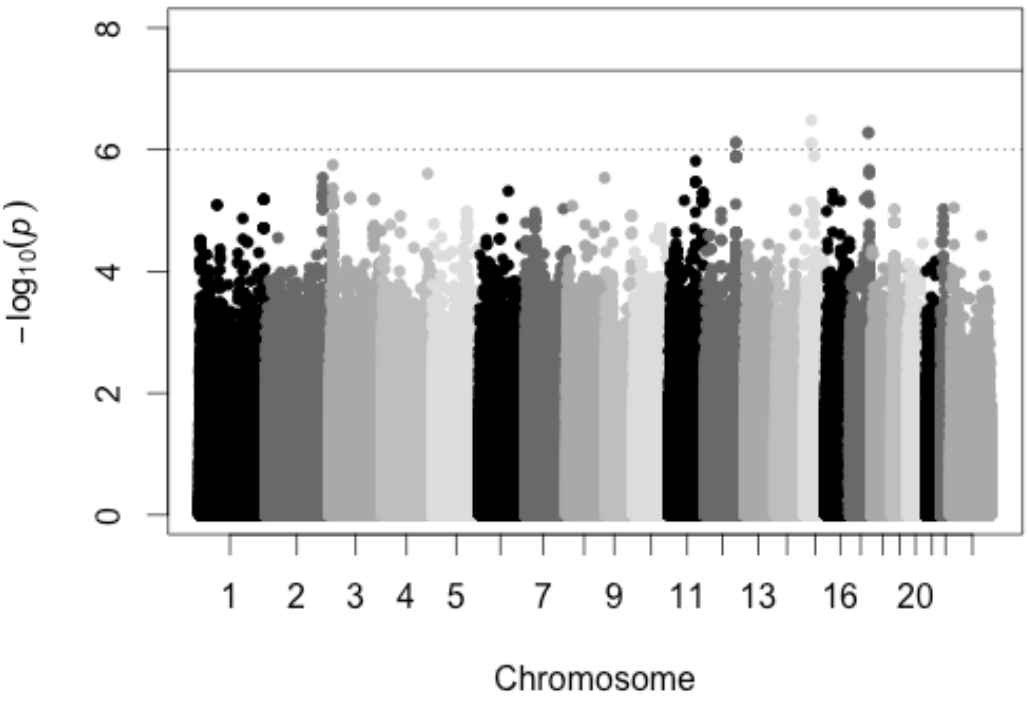

Figure 8: Manhattan Plot for Head BMC

For the head BMC phenotype, no significant signals were detected. Three total suggestive significance signals were observed.

Table 12: Suggestive Genes Associated with Head BMC

Suggestive SNP	Gene	Chr.	Suggestive P-value	Gene Function Relevance to Bone Biology
rs11858767	*SEMA6D*	15q21.1	3.274e-07	The SEMA6D gene is stimulated during osteoclast differentiation through the receptor complex Pleixn-A1/TREM-2/DAP12 in osteoclast cell precursors[130,131].
rs1495931	*FBXW8*	12q24.22	7.635e-07	The gene is expressed in embryonic bones. Null mutations resulted in small stature. It is suggested that *FBXW8* loss of function reduces the contribution of bone and muscle to the overall growth in mice studies[132]. Another study of 19,000 people of European ancestry found that this gene was associated with an increase in hippocampal volume, this study links this association with the head BMC phenotype[133].
rs1495931	*NOS1*	12q24.22	7.635e-07	A polymorphism in this gene was associated with BMD in Korean postmenopausal women in a previous study[134]. This variant was found to have a 3.7 fold higher prevalence of osteoporosis at the femoral neck.

3.2.4 Whole Body (Excluding Head) Bone Mineral Content GWAS

The Manhattan plot for the GWAS of whole body (excluding head) BMC is shown in Figure 9.

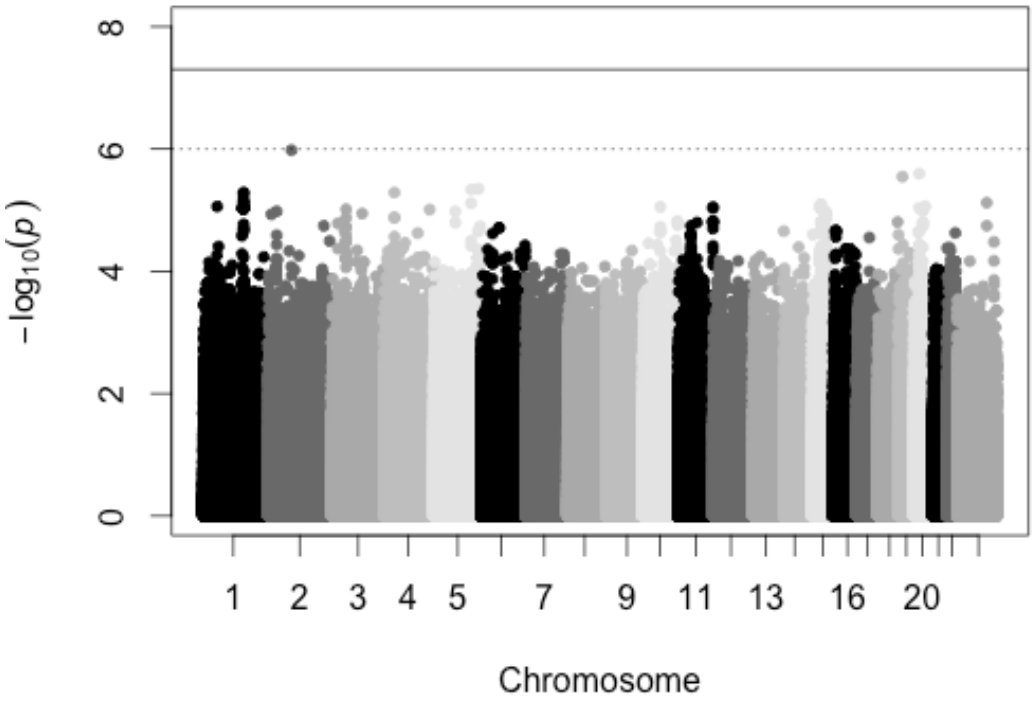

Figure 9: Manhattan Plot for Whole Body (Excluding Head) BMC

For the whole body (excluding head) BMC phenotype, no significant signals were detected. One suggestive significance signal was observed.

Table 13: Suggestive Genes Associated with Whole Body (Excluding Head) BMC

Suggestive SNP	Gene	Chr.	Suggestive P-value	Gene Function Relevance to Bone Biology
2-87831421	*RGPD2*	2p11.2	1.046e-06	The *RGPD2* gene promoter is a transcription factor binding site for *GATA-1* and *ER-alpha*[135].The *GATA-1* gene is believed to play a significant role in bone regulation as knockout studies have reported that mice deficient in GATA-1 may have the potential to rescue osteoporotic bone phenotype[136]. Estrogen receptors have been well studied in their impact on bone. The ER-alpha plays a vital role in mediating estrogen-dependent bone maintenance. It is believed that estrogen prevents bone loss via the ER-alpha in osteoclasts[137]. Additionally, ER-alpha has been observed in osteoblasts and osteocytes, predominantly in cortical bone[138,139].
2-87831421	*CD8A*	2p11.2	1.046e-06	The *CD8A* gene promoter is a transcription factor binding site for *STAT1*[140].
2-87831421	*PLGLB2*	2p11.2	1.046e-06	The *PLGLB2* gene promoter is a transcription factor binding site for *FOXO1*[141]. The *FOXO1* gene is a positive regulator of bone formation in osteoblasts. This transcription factor has been found to be central to an intricate process that translates the signal of oxidative stress and serotonin to regulate bone remodeling[142]. In mice, *FOXO1* knockout has a severe effect on skeletogenesis and craniofacial development[143,144].

3.3 BONE GEOMETRY GWAS

The significant and suggestive signals detected through the GWAS of femoral neck cross-sectional area, section modulus, and width are presented in Table 14.

Table 14: GWAS Findings for Bone Geometry Phenotypes

Phenotype	Number of Significant Loci	Number of Suggestive Loci	Implicated Genes
Femoral Neck Cross- Section Area	1	2	*TRAT1*
Femoral Neck Section Modulus	2	8	*HOXD* Cluster *NAV3*
Femoral Neck Width	0	3	---------

3.3.1 Femoral Neck Cross-Sectional Area GWAS

The Manhattan plot for the GWAS of femoral neck cross-sectional area is shown in Figure 10.

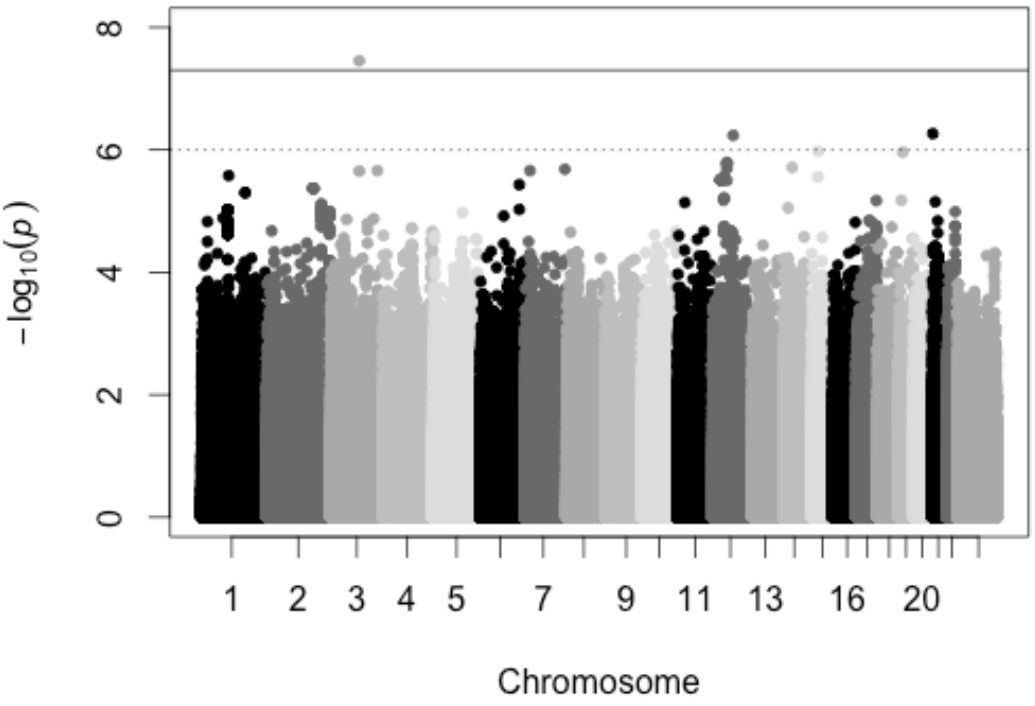

Figure 10: Manhattan Plot for Femoral Neck CSA

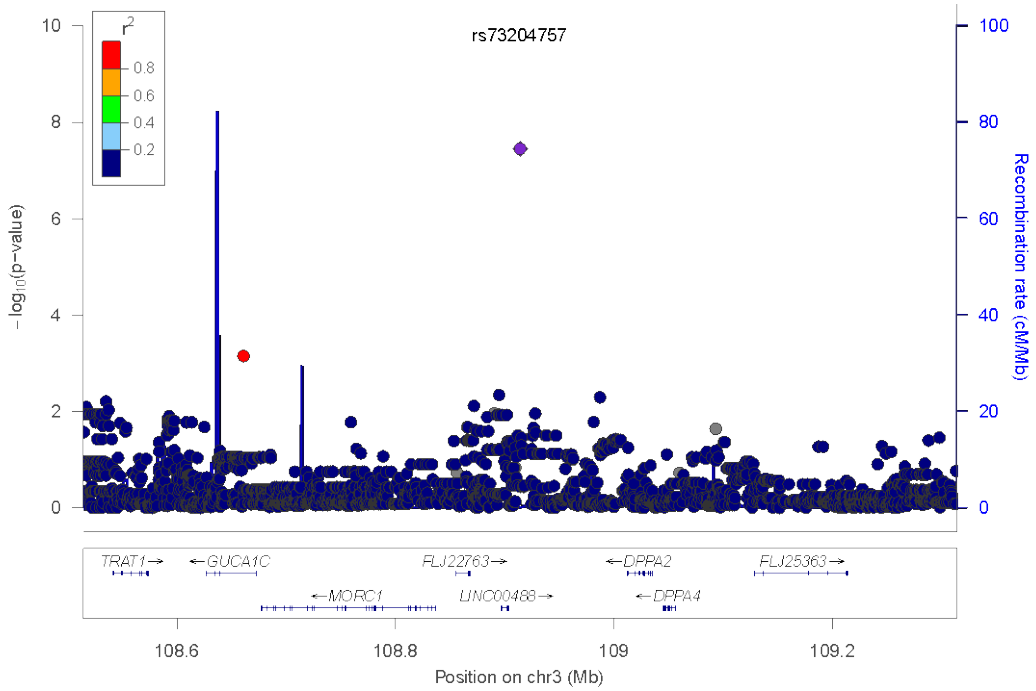

Figure 11: LocusZoom Plot for Significant Loci Associated Femoral Neck CSA

Annotation of the genes surrounding significant (P-value = 3.512 x 10^{-8}) signal rs73204757 on chromosome 3 suggests that the *TRAT1* gene (about 350kb upstream of the SNP) has the strongest relation to bone biology. However, the *TRAT1* gene promoter is one of the transcription factor-binding sites for STAT3 transcription factor[145]. STAT3 is expressed in bone and joint cells, including osteoclasts, osteocytes, osteoblasts, and chondrocytes. The STAT3 transcription factor is activated by a variety of growth factors and cytokines that regulate the differentiation of osteoblast and osteoclasts as well as the proliferation of chondrocytes. Additionally, STAT3 was found to be responsive to mechanical stimulators and may play a role in the mechanical signal transduction of bone[146]. Hyper IgE syndrome is caused by loss of function mutations in the *STAT3* gene. Patients with this genetic condition frequently experience osteopenia and pathologic fractures. Further, mice deficient in STAT3 have increased osteoclast activity yieled a decrease in bone mass[147]. This previous research could suggest that the *TRAT1* gene might be involved in the

tightly regulated system of transcription factors that are necessary bone development and homeostasis. While this is a weaker biological story that other implicated genes, it is possible that genetic variation in the *TRAT1* promoter could alter expression and activity of the STAT3 transcription factor.

Two suggestive significance signals were observed for the femoral neck cross-sectional area phenotype. Of particular interest is that loci including the *NAV3* gene reached suggestive loci. This loci reached GWAS significance for the femoral neck section modulus phenotype and was found to have relevance to bone homeostasis because the *NAV3* promoter includes a transcription factors binding site for STAT1 transcription factor, a well-studied regulator of osteoblast proliferation[148].

Table 15: Suggestive Genes Associated with Femoral Neck CSA

Suggestive SNP	Gene	Chr.	Suggestive P-value	Gene Function Relevance to Bone Biology
rs77689273	*NAV3*	12q21.2	5.795e-07	The *NAV3* gene promoter is a transcription factor binding site for the *STAT1* transcription factor[149].
21-9935897	*TPTE*	21p11.2	5.427e-07	The TPTE gene is involved in the signal transduction pathways of the endocrine function of the testes. This gene is overexpressed in bone and testes[150].

3.3.2 Femoral Neck Section Modulus GWAS

The Manhattan plot for the GWAS of femoral neck section modulus is shown in Figure 12.

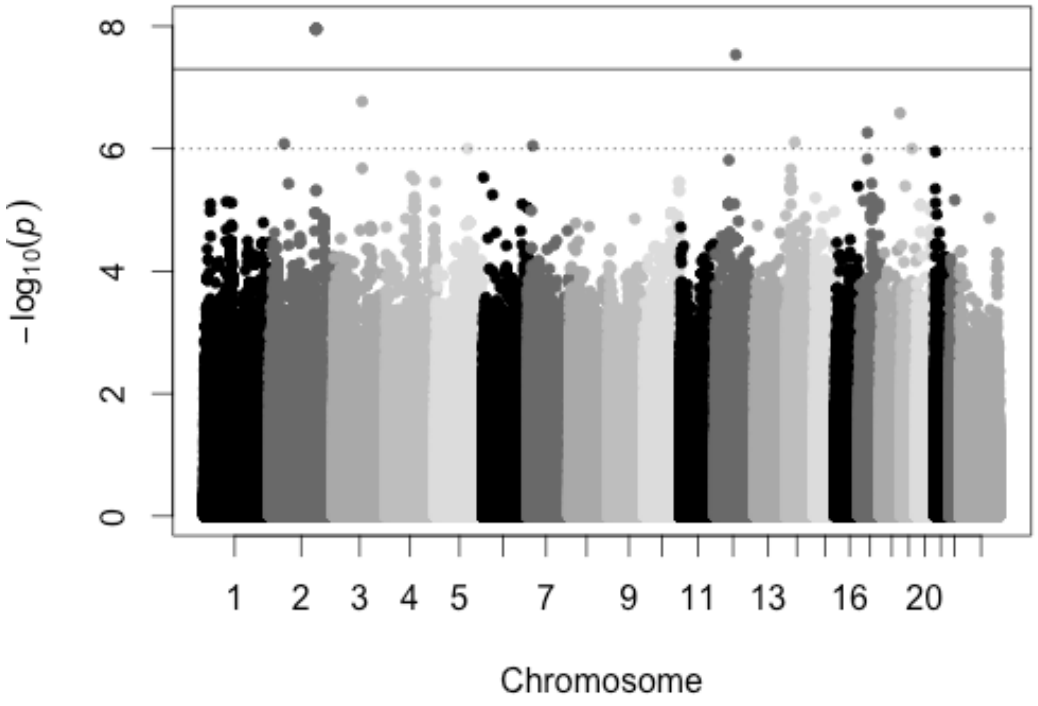

Figure 12: Manhattan Plot for Femoral Section Modulus

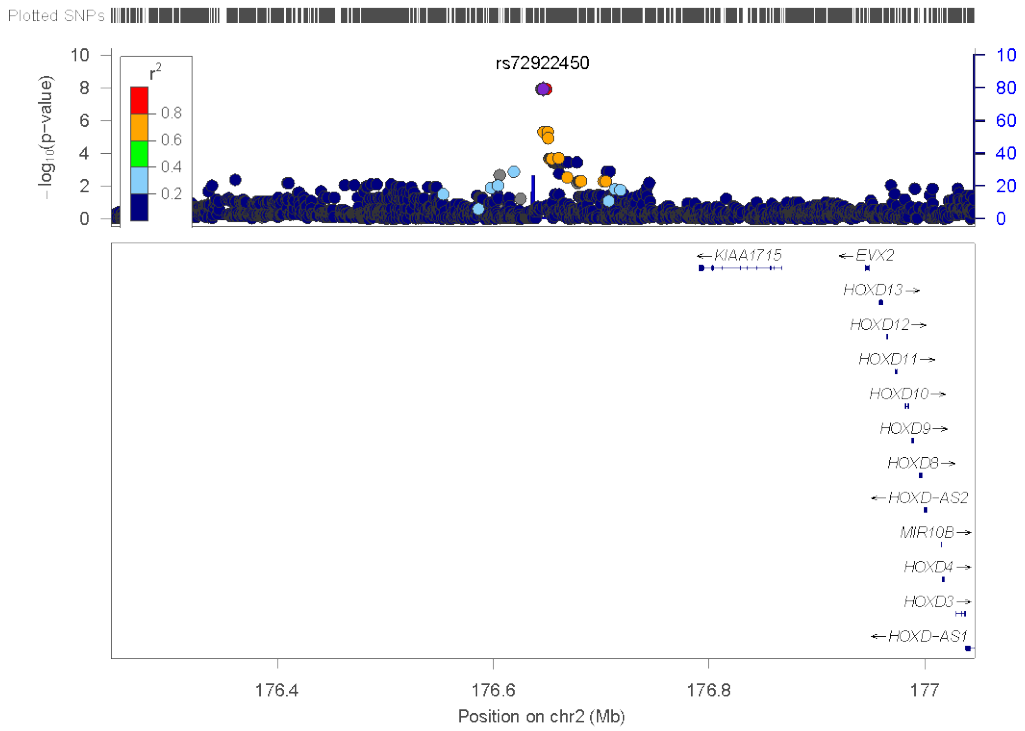

Figure 13: LocusZoom Plot for Significant Loci Associated with Femoral Neck Section Modulus

Two signals reaching genome-wide significance were detected for the femoral neck section modulus phenotype. The locus indicating the strongest evidence of association (P=1.11E10-8) was identified as the region of chromosome 2q31.1 and contains a homeobox D cluster of genes. The homeobox genes encode a highly conserved family of transcription factors are involved in limb development and skeletal differentiation. Previous studies have shown that deletions of the HOXD gene cluster result in a dominantly inherited disease resulting in synpolydactyly and lib malformations[151]. Cartilage and bone defects have also been characterize in detail with this deletion[152]. The first phase of HOXD gene expression is necessary for the forearm and upper arm long bone development. In later stages HOXD genes also play a crucial role in the development of the vertebrae[153]. Previous studies have implicated them in the specification of positional identity, as growth regulators, and involved in the timing of differentiation[154]. Further HOXD-13 mutations result in the shortening of the long bones, including the femur, tibia, fibula, and the tarsometatarsals. Targeted disruption of HOVD-10 produced mice with hindlimb defects in gait and adduction due to alterations in the vertebral column and in the bones of the hindlimb[155]. Other studies targeting the HOXD-3 gene demonstrated that mutations of the gene produced mice with radically remodeled skull[156]. Many functional animal studies have proven this cluster of genes essential in bone development.

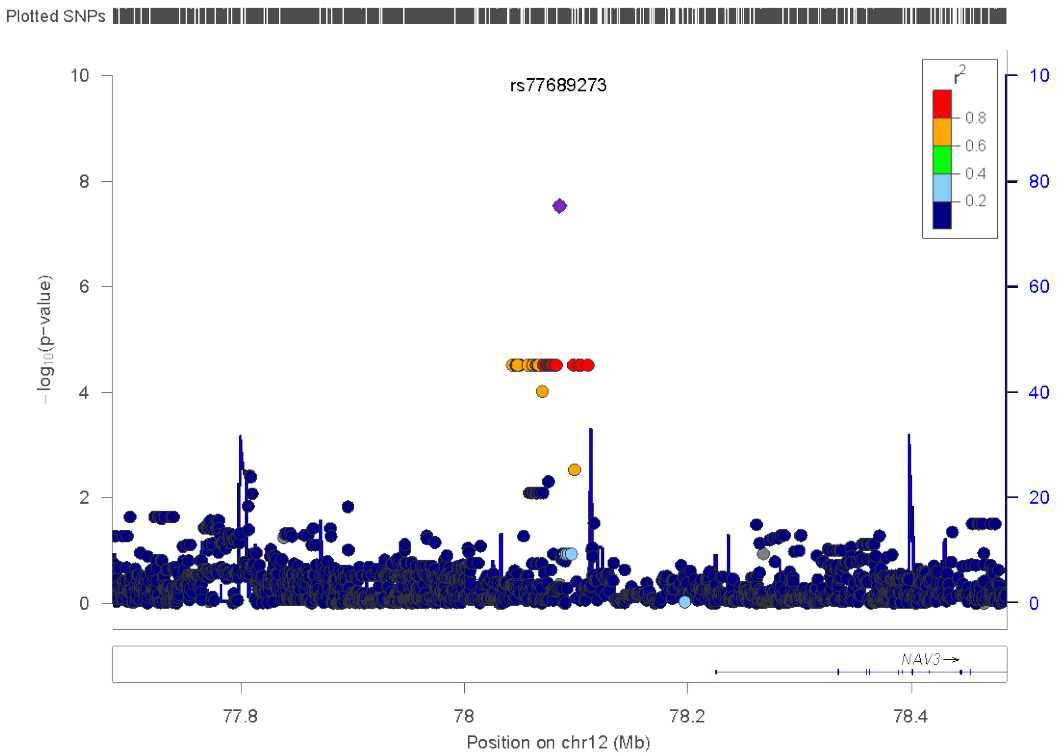

Figure 14: LocusZoom Plot for Significant Loci Associated with Femoral Neck Section Modulus

The second significant signal was identified as, rs77689273, which neighbors the *NAV3* gene. This gene is a member of the neuron navigator family and is primarily expressed in the nervous system[157]. However, the *NAV3* gene promoter is a transcription factor-binding site for STAT1 transcription factor[149]. Studies have shown that STAT1 is an inhibitor of osteoblast and osteoclast differentiation. Mice deficient in STAT1 were shown to have increased osteoclast activity, despite showing increased BMD and increase bone formation. These results implicate that STAT1 is involved in multiple pathways that independently regulate bone homeostasis[148]. The *STAT1* gene is a critical regulator for both osteoclastogenesis and osteoblast differentiations in skeletal fracture healing. Again, while this is a weaker biological story that other implicated genes,

it is possible that genetic variation in the *NAV3* promoter could alter expression and activity of the STAT1 transcription factor.

Eight total suggestive significance signals were observed for the femoral neck section modulus phenotype. Genes found to have relevance to bone regulation are listed below.

Of particular interest is the region including the *TRAT1*, which was implicated with the femoral neck CSA phenotype and reached suggestive significance for the femoral neck section modulus phenotype. Additionally a loci on chromosome 14 was determined to have suggestive significance, this region includes the *BMP4* gene. This gene is a highly conserved, well-studied candidate gene that induces cartilage and bone formation[158]. BMP4 also binds with BMPR1A, which is located at another suggestive locus associated with femoral neck width. This complex induces osteoblastogenesis and regulates the expression of the *SOST* gene, which encodes sclerosin, a key regulator of bone homeostasis[159].

Table 16: Suggestive Genes Associated with Femoral Neck Section Modulus

Suggestive SNP	Gene	Chr.	Suggestive P-value	Gene Function Relevance to Bone Biology
rs470186	*GALR1*	18q23	2.616e-07	Functional studies of GALR1 found that the distribution of GALR1 was more extensive in reserve zone chondrocytes. The concentration of ligand for this receptor, galanin, was significantly increased in rats with rib fracture. This study strongly implicated Galanin and GALR1 as involved in cartilage growth plate physiology and fracture repair[160].
rs1583390	*ASIC2*	17q12	5.462e-07	Found to be abundant in specialized human bone cells, this gene was nominated as a candidate gene for sensing and regulating bone balances serum pH variations. pH sensing Acid- sensing channels 2 (ASIC2) are found in both osteoblasts and osteoclasts and may explain how bone cell function can be modulated by environmental pH under physiological and pathological conditions[161].
rs72915032	*ACYP2*	2p16.2	8.288e-07	The ACYP2 gene is involved in hydrolysis of phosphoenzyme intermediates of different membrane pumps, particularly the Ca2+/Mg2+-/ATPase from sarcoplasmic reticulum of skeletal muscle[162]. This loci was identified as significant in previously published GWAS studies on the genetic determinants of heel bone properties, bone mineral density, and risk of fractures[163].
rs73204757	*TRAT1*	3q13.13	1.687e-07	The *TRAT1* gene promoter is a transcription factor binding site for the *STAT1* gene[145].
Rs73294246	*CSNK1G3*	5q23.2	9.949e-07	Identified in previously published association study of blood pressure and bone mineral density[164].
rs111708633	*BMP4*	14q22.2	7.829e-07	The BMP4 is a highly conserved member of the bone morphogenetic protein family, a family that stimulates growth and differentiation factors. The gene induces cartilage and bone formation. The BMP4 gene also acts in limb formation, tooth development, mesoderm induction, and fracture repair. A reduction in expression has been linked with a number of bone diseases including Fibrodysplasia Ossificans Progressiva, a dominantly inherited disorder of aberrant joint formation and heterotropic bone formation[158,165–169].

3.3.3 Femoral Neck Width GWAS

The Manhattan plot for the GWAS of femoral neck width is shown in Figure 15.

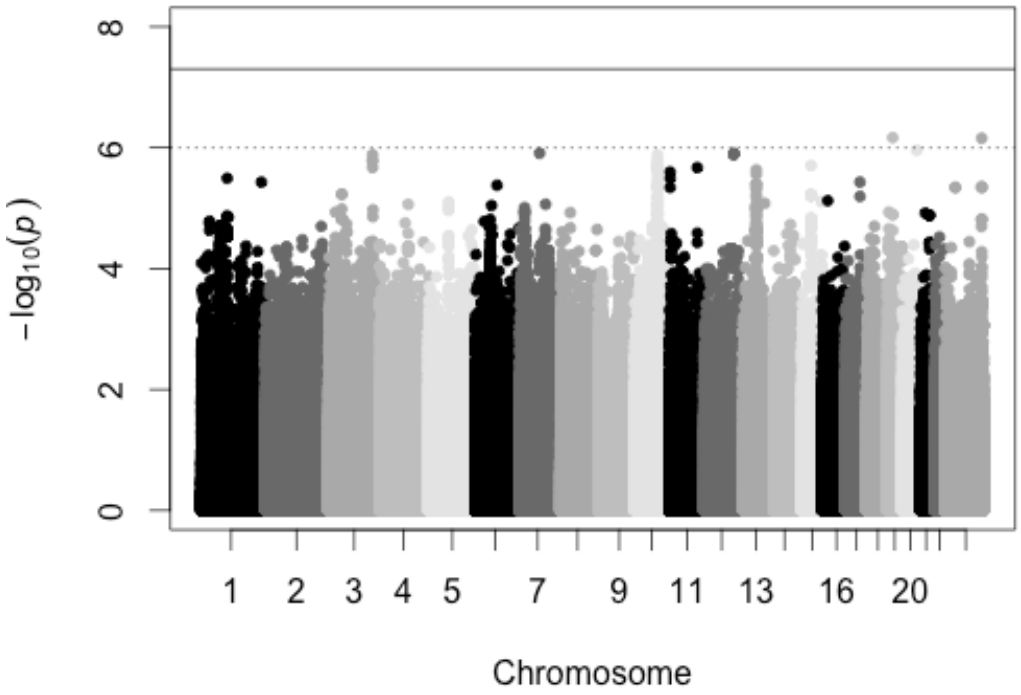

Figure 15: Manhattan Plot for Femoral Neck Width

For the femoral neck width phenotype, no genome-wide significant signals were detected. Three suggestive significance signals were observed for the femoral neck width phenotype. A SNP neighboring the *BMPR1A* gene was found to have a suggestive association. The *BMPR1A* gene interacts with many candidate genes of bone biology and many previous studies have demonstrated the impact BMPR1A has bone osteoblast activity, bone turnover, and age dependent bone development[170].

Table 17: Suggestive Genes Associated with Femoral Neck Width

Suggestive SNP	Gene	Chr.	Suggestive P-value	Gene Function Relevance to Bone Biology
rs12982091	*ZNF98*	19q12	6.812e-07	The *ZNF98* gene promoter is a transcription factor binding site for *GATA-1*[171].
rs75188850	*BMPR1A*	10q22.3	1.316e-06	The *BMPR1A* gene encodes the bone morphogenetic protein receptor which ligands to the transforming growth factor beta (TGF-β) pathway. When the *BMPR1A* / TGF-β complex is bound the SMAD protein complex is activated. Research investigating osteoblast-specific disruption of the *BMPR1A* gene yielded normal numbers of osteoblasts, however, irregular calcification, low bone mass, and reduced bone resorption and bone turnover. Studies have demonstrate critical and age-dependent roles for BMP signaling by *BMPR1A* in osteoblasts for bone remodeling. Similarly, deletion of *BMPR1A* gene in osteoclasts increases osteoblastic bone formation, suggesting that *BMPR1A* signaling negatively regulates osteoclast differentiation. Soluble BMPR1A protein inhibitor is now being researched as a therapy to stimulate bone formation and restore bone mass in mice with low BMD[170,172,173].
rs75188850	*MMRN2*	10q22.3	1.316e-06	The *MMRN2* gene promoter is a transcription factor binding site for the *STAT1* and *PPAR-Gamma-1* genes[174].
rs75188850	*SNCG*	10q22.3	1.316e-06	The *SNCG* gene promoter is a transcription factor binding site for the *PPAR-Gamma-1* gene[175].
rs75188850	*FAM25A*	10q22.3	1.316e-06	The *FAM25A* gene promoter is a transcription factor binding site for the *GATA-1, FOXO1A* and *ER-alpha* genes[176].
rs75188850	*AGAP11*	10q22.3	1.316e-06	The *AGAP11* gene promoter is a transcription factor binding site for the *STAT1* and *PPAR-Gamma-1* genes[177].
rs75188850	*GLUD1*	10q22.3	1.316e-06	The *GLUD1* gene promoter is a transcription factor binding site for the *ER-alpha* gene[178].
rs75188850	*MINPP1*	10q22.3	1.316e-06	The *MINPP1* gene encodes an enzyme that removes 3-phosphate from inositol phosphate substrates in order to regulate cellular levels of inositol pentakisphosphate. This conserved gene may play a role in bone development, specifically endochondral ossification. Although Minpp1 expression is highly upregulated during endochondral ossification, normal chondrocyte differentiation and longitudinal bone development were observed in Minpp1-deficient mice[179].

4.0 DISCUSSION

Osteoporosis is a serious public health concern that results in increased bone fragility and risk of fracture for millions of individuals in the U.S. Though current research has focused primarily on bone health in the elderly, early bone health, including peak BMD attainment, is the strongest determinant of bone health later in life. Twin and family studies have consistently established a strong genetic component in peak BMD, though the specific genes influencing variation in bone development are largely unknown[180]. Moreover, the question of whether the genes influencing bone health during childhood are the same as those influencing bone health later in life is currently unknown. In this study we performed ten separate GWAS scans to detect genetic variants associated with ten different bone phenotypes including site specific bone mineral density, bone mineral content, and bone geometry measurements. We successfully identified five GWAS significant loci and nominated many potential bone specific genes. Implicated genes are believed to be involved in a variety of possible processes such as embryonic bone development, bone remodeling, and fracture repair, which is consistent with the prevailing view that the genetic etiology of bone health includes many genes acting through multiple pathways.

This study is one of the first studies to determine genetic associations with childhood bone health. We successfully identified four novel, significant genomic loci and overall, 30 loci significantly or suggestively associated with childhood bone health. Our hypothesis generating investigation of common variants of the genome provides novel insights into bone biology, implicating several genes clustering in pathways influencing bone development.

Our results highlight the polygenic nature underlying bone development and variation in bone health and the essential role of several biological pathways that influence osteoporosis risk.

Pathways have been constructed, utilizing previous literature, in order to propose interactions between the genes implicated in this study and well known candidate genes. Further these pathways illustrate how changes or variations in our implicated genes can lead to changes in bone health during development.

Below is a table of well-known candidate genes of bone biology that are believed to also be interacting with the implicated genes of this study.

Table 1: Candidate Genes of Bone Development for Previous Literature

Candidate Gene	Proposed Function
RANK/RANKL	RANK/RANKL signals the formation of osteoclast precursors, activation, and survival in normal bone remodeling [5,26,27]. Mice deficient in RANKL and RANK develop osteopetrosis because of the inability to form osteoclasts[28,29].
OPG	This gene acts as a negative regulator of bone resorption. Overexpression blocks osteoclast formation inducing osteopetrosis. Gene knockout results in enhanced bone remodeling and bone loss, resulting in osteoporosis[5].
COL1A1/2	Encode matrix proteins. The expression of these genes and the balance of collagen fibrils are essential for proper formation of bone. Poor collagen quality results in reduced bone strength. Mutations in the *COL1A1* gene results in osteogenesis imperfect, a genetic condition with a phenotype of extremely severe osteoporosis[8,20,21].
LRP5	A receptor for canonical Wnt signaling. Wnt signaling is involved in processes including apoptosis, limb development, and osteoblast and chondrocyte differentiation. Knockout mice have shown low bone mass is a result of decreased osteoblast proliferation[13,30].
TGF-β1	Abundant in bone matrix, the released TGF-B1 protein is an essential controller of osteoblast proliferation and differentiation. Gene knockout results in osteopenic phenotype[31].
Osterix	Transcriptional regulator expressed in chondrocytes and in osteoblasts. Targeted inactivation of this gene led to the complete absence of bone synthesis throughout the skeletal and a loss of most markers of bone differentiation[20].

Table 1 Continued

VDR	The first candidate gene to be investigated in the osteoporosis field, the *VDR* gene encodes the vitamin D receptor, which allows the body to metabolize vitamin D [21].
ESR1	Encoding estrogen receptor, believed to have a role in modulating osteoclast differentiation and function. Several polymorphisms in this gene have been connected with the rate of bone loss after menopause[20].
TNF-α	TNF-alpha is believed to reduce osteoblast mediated mineralization while simultaneously inducing osteoclast differentiation [32,5].
RUNX2	Key transcription factor that induces proliferations and differentiation into preosteoblasts and mature osteoblasts and therefore bone formation[2].
SOST	The *SOST* gene encodes the protein sclerostin in osteocytes. The main role of this protein is to inhibit bone formation by interfering with Wnt signaling. Sclerostin may also promote apoptosis in bone cells, further inhibiting bone growth.[21].
SOX9	A critical transcription factor for BMP1 induced chondrocyte differentiation and osteoblast activity[33].
SMAD1	This gene is believed to play a key role in bone development and postnatal bone formation. SMAD proteins control the expression of RUNX2[34].

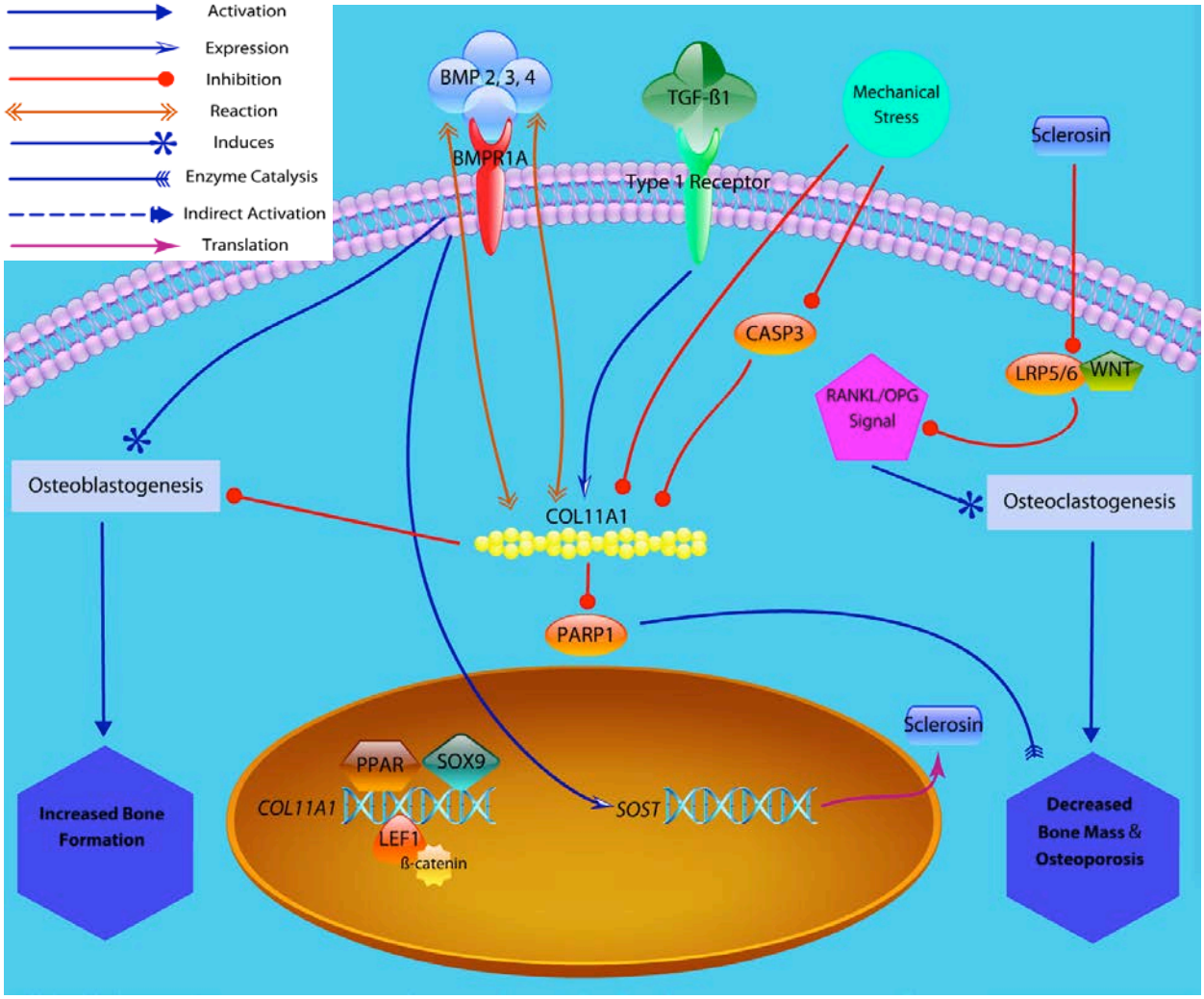

Figure 16: Pathway of COL11A1 Interactions

COL11A1 is directly and indirectly involved in numerous pathways throughout the cell. First, COL11A1 is believed to regulate osteoblastogenesis and terminal osteoblast differentiation. The protein also reacts with BMP2 and BMP4, key components in osteoblast production and the translation of sclerosin, which is a critical regulator of osteoblast activity. Mechanical stress is also hypothesized to inhibit collagen formation. COL11A1 inhibits PARP1, of which the enzymatic activity contributes to reduced bone mass. Lastly, *COL11A1* promoter is a transcription factor binding site for PPAR and SOX9. SOX9 is involved in the activation of COL2A1 and PPAR is involved in the regulation of *HOXD10* and *HOXD13*[123,125,181–192].

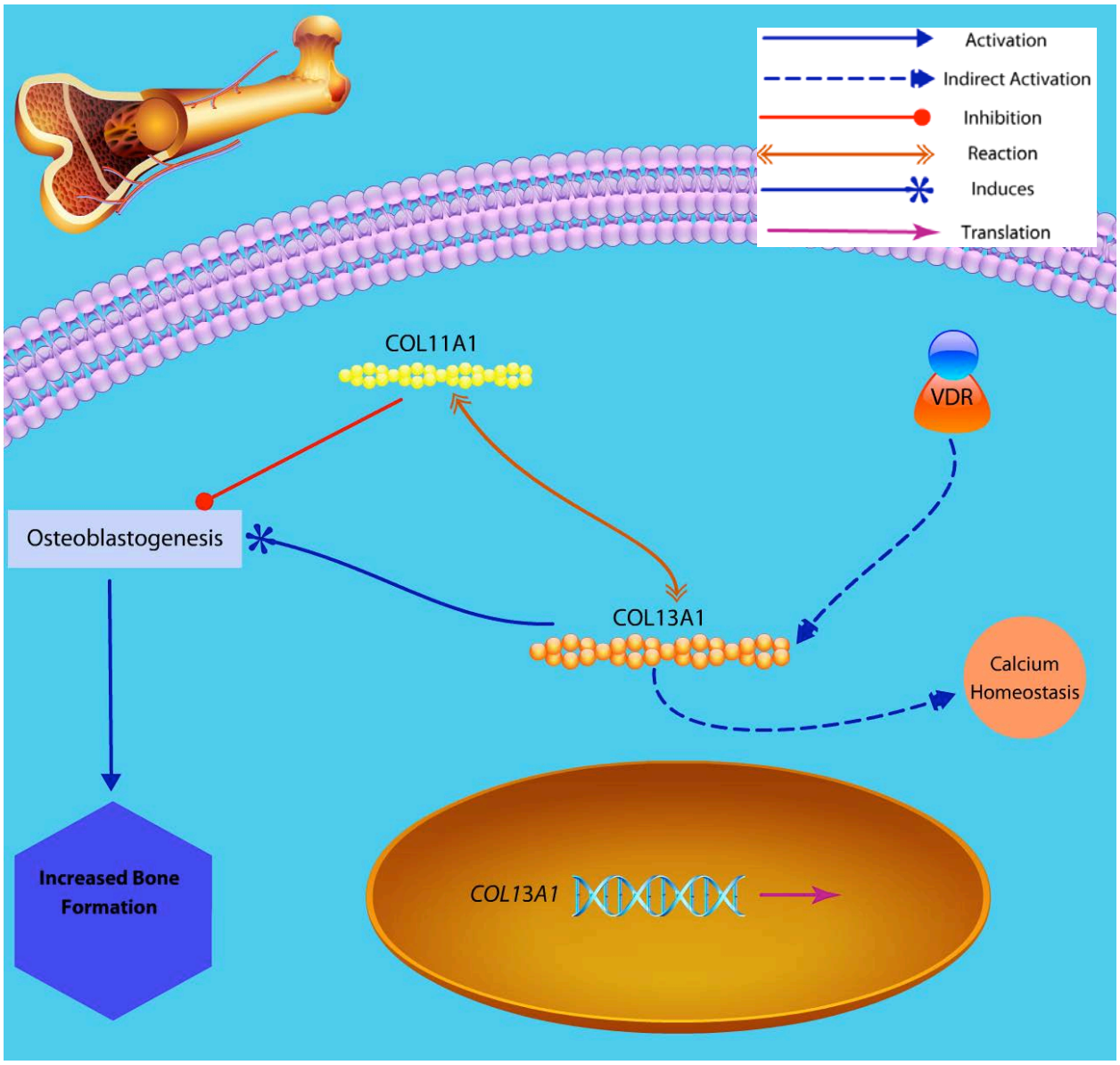

Figure 17: Pathway of COL13A1 Interactions

COL13A1 is indirectly involved with the Vitamin D receptor, a candidate gene in bone biology. COL13A1 is also believed to be indirectly involved in the calcium homeostasis process. COL13A1 interacts with COL11A, which as described above, inhibits osteoblast differentiation. COL13A1 may lead to osteoblast differentiation and therefore increase bone formation[97,99,193–195].

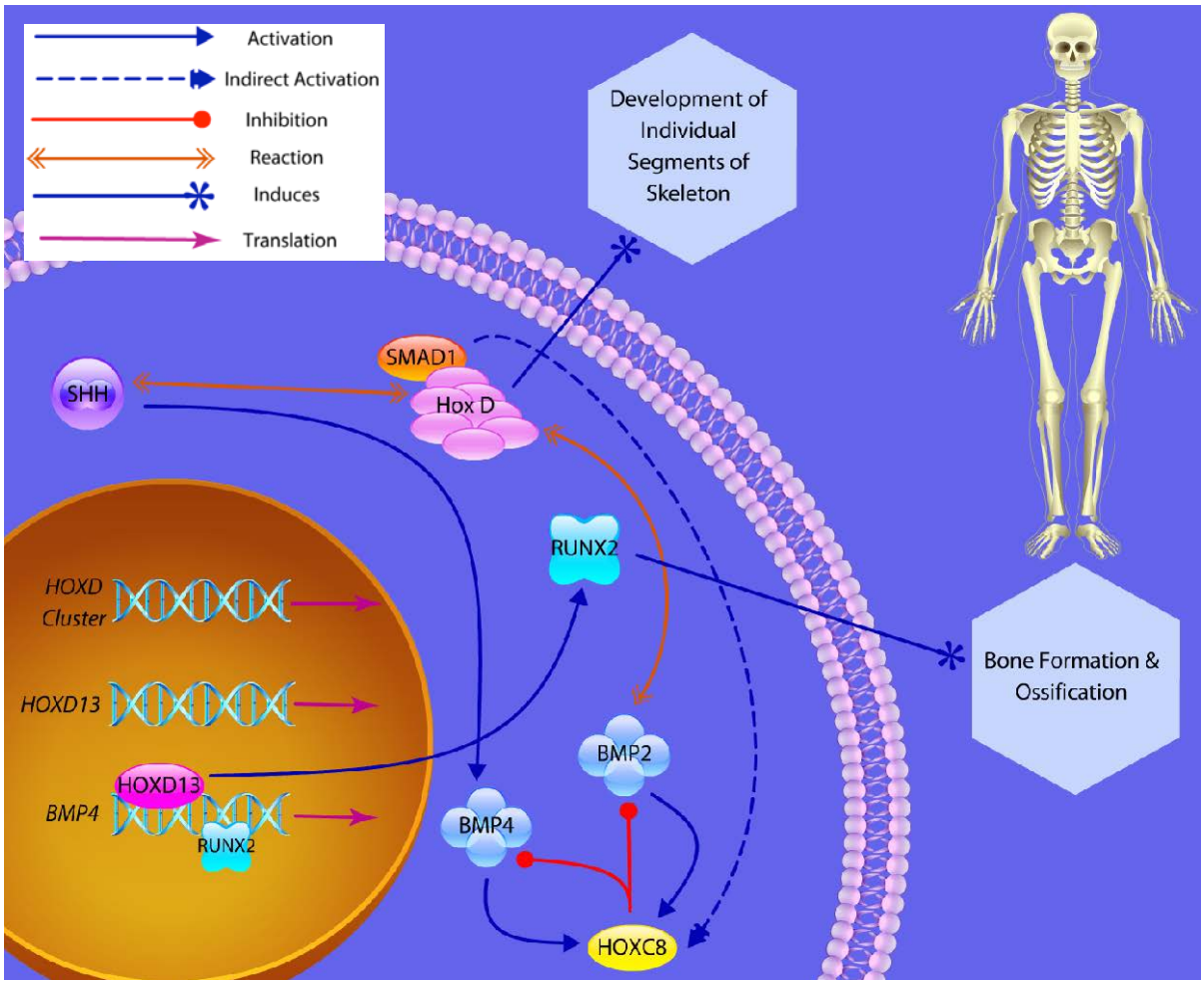

Figure 18: Pathway of HOXD Gene Cluster Interactions

The Homeobox D cluster of genes is an evolutionarily conserved family that is involved in limb development and skeletal differentiation. HOXD proteins form a complex with SMAD1 that impacts the expression of RUNX2, a vital regulator of osteoblast proliferation and differentiation, indirectly regulates BMP2 and BMP4, key components in osteoblast production, and reacts with SHH, known to enhance bone regeneration. *BMP4* gene protein also acts as a binding site for HOXD13 to influence RUNX2 expression and therefore bone formation[2,151,155,196–200].

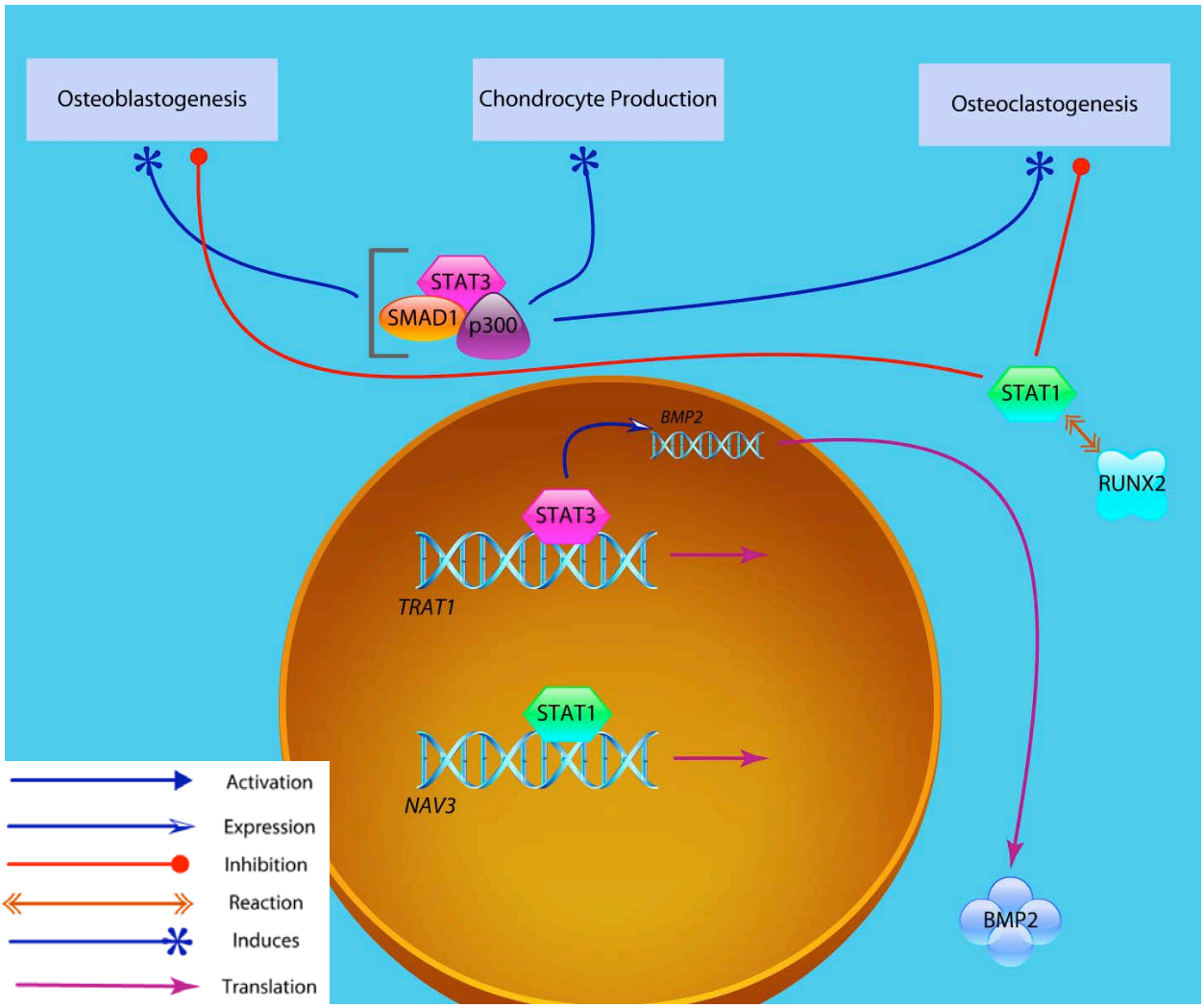

Figure 19: Pathway for *TRAT1* and *NAV3* Gene Interactions

The promoter of the *TRAT1* gene is a transcription factor binding site for STAT3. STAT3 activates BMP2, a key regulator of bone homeostasis. STAT3 also creates a complex with SMAD1 and p300, which can stimulate osteoblast activity, chondrocyte production, as well as osteoclastogenesis. The promoter of the *NAV3* gene is a transcription factor binding site for STAT1. STAT1 can negatively regulate osteoblast and osteoclast activity during bone development. STAT1 also reacts with RUNX2, which induces osteoblast differentiation[145,147–149,201]. While these two implicated genes have a weaker relevance and biological connection to

bone development compared to other nominated genes, these genes have the potential to impact bone variation.

Our study nominated five significant loci, four of these loci are novel and have not previously been implicated in GWAS studies of child or adult bone health. The significant signal for hip BMC observed for rs12564863 on chromosome 1q21 has previously been identified in a GWAS meta-analysis associated with BMD and risk of fracture in adults. These results suggest that the genetic contributors to bone health and normal bone variation may change in their regulation of bone development over time however, some candidate genes may play a significant role throughout the lifespan.

Overall, the results of this study forward our understanding of the genetic contributors to bone health early in life. Investigations, such as this, into the genetics of bone development in children may also yield insight into the genetic factors that contribute to bone health later in life. This research has major public health significance because this information may ultimately aid in determining if some individuals have a high genetic risk for poor bone health. This idea could lead to screening programs aimed at specifically identifying children that have a genetic predisposition or genetic risk factors for osteoporosis later life. In the high-risk children that may be identified, effective, targeted interventions could be implemented early in order to promote optimal bone health before peak bone mass is reached.

4.1 LIMITATIONS

While this study has many strengths, including well-defined bone phenotypes, high-quality GWAS data, and extensive gene annotation, our study has limitations. A major limitation is the lack of

replication with a small sample size. This issue is partly lessened by the compelling biological evidence of the implicated genes in mechanisms related to bone health. However, this study included 296 children with an average age of 5 years. Observed genetic associations need to be replicated in independent data sets. Additional research with a larger sample will allow our findings to be confirmed, identify any missed loci, and many give more information on the effect size of each variant. Due to the small sample size, this study suffered from low power to detect any particular variant, therefore we most likely missed other true associations. Areas of suggestive significance would also benefit greatly from a larger study to see if in fact associations with those loci are statistically significant.

Another potential limitation to this study is the possible influence of un-modeled sources of phenotype variation in the GWAS analysis. Covariates included age, height, sex, weight, and principle components. Future analysis could include the lifestyle data recorded for the children as part of the Iowa Bone Development Study. These lifestyle data include diet and exercise measurements. Because of the known impact of a balanced diet, including essential vitamins and minerals for bone development, and physical activity on bone health, these factors surely influence bone phenotype measurements. Including these data in our analysis may increase the power to detect genetic factors by reducing phenotypic variance due to environmental factors known to contribute to bone health. On the other hand, inclusion of additional covariates would consume additional degrees of freedom in the statistical model, thereby reducing power to detect genetic association. Each approach has costs and possible benefits, and it is not known whether or not adjustment for additional sources of phenotypic variance would ultimately benefit the study. Nevertheless, omission of lifestyle data or other un-modeled environmental sources of phenotypic variation would not bias the present analysis (i.e., would not cause false positive associations)

because the constitutional genome is inherited and therefore not susceptible to confounding by environmental factors.

Lastly, the SNPs identified in this study are not necessarily causal variants. It is likely that some or most of the SNPs identified are in linkage disequilibrium with the underlying causal variants. It was our goal to determine plausible causal genes underlying the GWAS signals. However, follow-up studies are needed to verify the nominated genes and to identify the causal variants. Ultimately, functional analysis of the genes and causal variants will be required in order to understand the biological mechanisms through which they exert their effects.

4.2 FUTURE WORK

Because there are still many unanswered questions and unknown factors in bone biology and specifically the genetic regulators that contribute to bone health, there are many possible future directions for this research. Possible directions are outlined below.

4.2.1 A Priori Research

As the GWAS has matured as an approach for studying complex disease, several methods have been proposed to more effectively utilize the wealth of data created. These methods include a priori information, such as gene expression and biological function[202]. While this information was included in this study by annotating top hits, future studies could include a more in depth analysis of a priori candidate gene information. This information will allow us to identify all of the previous implicated loci and candidate genes related to bone health and determine the significance of those

genetic variants with our GWAS data. This method not only allows us to analyze the significance of well-known candidate genes in child populations but also acts as positive control measure. If we are able to confirm associations with known candidate genes, then we can interpret our findings of novel loci and implicated genes with more confidence.

4.2.2 Genetic Risk Score

While GWAS is hypothesis generating and aiming to determine genes associated with variations in bone health, future work could incorporate this information to predict the risk of poor bone health and osteoporosis later in life. Several recent publications have utilized loci associated with adult bone mineral density to develop a genetic risk score algorithm that calculates the number of genetic risk variants for bone health. These genetic risk scores were then used to investigate the effect of reported GWAS-implicated BMD variants in children. These studies found that a higher genetic risk score (associated with a higher number of BMD-lowering variants) was negatively associated with BMD and BMC during childhood and adolescence and was associated with a slower rate of bone accrual during adolescence[36]. Rather than detecting single variants, with potentially small effect sizes, associated with poor bone health to identify children with a genetic predisposition to osteoporosis, calculating genetic risk scores could be more effective.

Future analysis could use this study's genome-wide significant loci and suggestive variants to develop a genetic risk score. Statistical analysis would determine if bone phenotype measurements were significantly different with a corresponding genetic risk score.

4.2.3 Longitudinal Data

This study focused on bone health for one target age, with the average age of 5 years, ranging from 4 to 7 years old. Future studies could include GWAS performed on the same bone phenotypes at several different ages of these children. As the Iowa Bone Development Study is a longitudinal study, this information would be readily available. This research could be repeated utilizing bone measurements at ages 11, 15, and 20 to determine if the genetic variants with a strong significance have the same the same significance at different ages at bone accrual rates. This information would shed light on the tightly regulated genetic pathways that influence bone health throughout the childhood and adolescence and the possible differences of genetic factors at different developmental periods. Moreover, longitudinal analysis methods, that simultaneously model the trajectory of bone phenotypes over time, could also be used for gene-mapping of the changes in bone health from childhood to adolescence to adulthood.

4.3 CONCLUSIONS

In summary, this study aimed to identify genetic variants that contribute to the variation in bone health in children. Research on the genetics of bone health has predominantly been performed in adult populations, however, it is our hypothesis that the genetic regulators of bone health may be stronger in childhood during a higher amount of bone production, bone accrual, and in the lack of many environmental factors that influence poor bone health in adults. To identify variants and genes implicated in childhood bone health, we performed separate genome-wide association studies for ten bone health phenotypes. Five genome-wide significant ($P \leq 5 \times 10^{-8}$) and twenty-

eight suggestive ($P<10^{-6}$) loci were identified in total. Associated loci included several genes with plausible roles related to bone health, such as *COL11A1, COL13A1, TRAT1, NAV3,* and a *HOXD* gene cluster. Implicated genes may represent significant roles in the converging pathways that regulate BMD, embryonic bone development, and bone remodeling. Furthermore, understanding the genetic determinants of bone health during childhood may have implications across the lifespan. Though osteoporosis is usually viewed as an age-related disorder, risk of osteoporosis is impacted by events occurring much early in life, including phases of bone mineral acquisition during youth. Therefore, identifying the genetic contributors for early skeletal health, such as genes implicated in this study, may ultimately lead to screening programs to identify children with a genetic risk factors for poor bone health and targeted interventions for those at high risk, to optimize bone health in adolescence, promote management of bone health across the lifespan, and lower risk for osteoporosis later in life.

5.0 PUBLIC HEALTH APPLICATION

As the knowledge of bone biology and the genetic contributions of bone health increases, efforts must be made to incorporate this understanding into the clinical realm. Incorporating genomics into clinical and public health practice is a way to identify high-risk individuals, emphasize prevention of disease, and promote the overall health of the community. If genetic risk factors for osteoporosis and poor bone health can be identified then a population-based health intervention could be implemented to identify children with a genetic predisposition in order to maximize the benefits of nutritional and physical interventions to optimize peak bone mass achieved during young adulthood. This chapter aims to propose a pilot childhood bone health screening program and intervention program for individuals found to be at higher risk.

Bone health is particularly amenable to a population- and community-based intervention because 1) bone fractures and osteoporosis affect a large portion of the population, 2) there is a widespread lack of knowledge about osteoporosis prevention and when peak bone mass is acquired, 3) state and local governments have incentives to promote this approach due to the cost of treating bone disease, and 4) the benefits of community based interventions extend to other areas of health, including diet and physical activity[203,204].

5.1 PREVIOUS EXAMPLES OF PUBLIC HEALTH GENETIC SCREENING PROGRAMS

Public health genetic screening programs have been utilized for decades in order to identify patients and families at risk for genetic conditions in order to change medical management and optimize the prognosis. The most prominent example of public health genetic screening is Newborn Screening (NBS). NBS screens for genetic conditions that negatively impact a child's long-term health or survival. Early detection, diagnosis, and treatment can prevent death or disability in identified children and can enable children to reach full potential. NBS identifies more than 6,000 babies with genetic conditions each year and represents the "public health success story of this decade[205]." The Recommended Uniform Screening Panel (RUSP) must approve conditions screened for with NBS. Evaluation for additions to NBS is based on a set of criteria that includes the natural history of the disorder, availability and accuracy of screening, availability of treatment, cost- effectiveness, clinical validity and utility, and time-sensitive nature[206]. This set of criteria is used as a benchmark for many public health programs to determine need and effectiveness. More evidence is needed to prove that a bone health screening program meets this set of criteria. However, NBS provides a successful example for future public health screening programs with the goal of early intervention and treatment in order to benefit long- term health.

Another recent public health genetic screening initiative includes Familial Hypercholesterolemia (FH) screening. FH is an autosomal dominant genetic condition characterized by abnormally high concentrations of LDL cholesterol, which causes a predisposition to premature heart disease and death. FH is one of the most common inherited disorders, and early diagnosis can initiate life style changes and the most effective disease management earlier. Overall, estimates are that less than 25% of those affected with FH are

diagnosed and the majority remains untreated or improperly treated. FH screening, through cholesterol screening, is a key public health program that aims to identify patients with FH, begin proper medical management, improve prognosis, reduce disease costs, and extend the program to cascade screening in order to other family members with this genetic risk factor to heart disease[207]. A recent meta-analysis of published data on cholesterol levels in FH individuals showed that when LDL was measured between 1 year and puberty, 96% of those with FH were detected with a false-positive rate of 1%[208]. Due to the evidence of early identification and management, the American Academy of Pediatrics (AAP) has recently cholesterol screening between the ages of 9 and 11 years to the schedule of screening and assessments for well-child visits[209]. FH screening is a nice parallel for bone health screening, specifically in children, because FH and osteoporosis do not necessarily cause serious health concerns from an early age, but early management and prevention can prevent serious outcomes later in life.

Lastly, another public health screening program that could serve as an example for a proposed bone health-screening program is universal immunohistochemistry (IHC) screening of all colon and uterine tumors. Universal screening of all newly diagnosed colon and uterine cancers through immunohistochemistry is a way to identify patients that may have Lynch syndrome, one of the most common hereditary cancer syndromes, in which 95% of affected individuals are undiagnosed[210]. IHC screens for a germline mutation in a mismatch repair gene which causes increased lifetime risk for cancers including colon, uterine, stomach, ovarian, bowel, as well as other types[211]. Diagnosis, through gene sequencing following screening, of this condition through universal screening can give insight into the potential risk for other types of cancer for the patient and family members, allowing for increased screening and possible surgeries to either prevent cancer or detect cancer early. While this screening program is different from the previous examples

because it is a screening tool for individuals that have already developed cancer, it is useful in identifying individuals with a single gene disorder that can be affected by environmental influences and early medical management.

NBS, FH, and Lynch syndrome screening are all included on the Centers for Disease Control and Prevention Office of Public Health Genomics Tier 1 List of genomic applications. This department aims to provide "timely and credible information for the effective and responsible translation of genome- based discoveries into public health and health care"[212]. A tiered system is used to distinguish between methods and tests of sufficient evidence of validity, clinical utility, promising evidence, and insufficient evidence. Tier 1 is the highest level of evidence, in which these tests inform are required to inform medical management, testing is covered by insurance, and clinical practice guidelines are based on systematic review supporting these tests. A genetic screening program for bone health in children would currently most likely fall under the Tier 3 level. This is because there is insufficient evidence of cost and clinical effectiveness. In order to obtain evidence, pilot studies need to be initiated, studying the best protocol for a program such as this[212].

There are many examples of public health genetic screening programs that provide models to build a bone health genetic screening program off of. These past examples include a focus on health promotion and disease prevention through the ability to identify individuals suspected of strong genetic risk factors in order to begin interventions and treatment early to in turn reap the most benefits and optimize health prevention later in life. These programs also have a strong emphasis on the implications of other family members and cascade family strategies. The core goals of these past programs can be easily applied to childhood bone health and implementing a

genetic screening program, bringing the understanding of the genetics of bone health into the practice and demonstrating the clinical validity of this information.

5.2 PROPOSAL FOR BONE HEALTH SCREENING

While there are still many unanswered questions regarding the genetics of bone biology and the variation in bone health, the increase in understanding in the field has increased dramatically in the past decade and could be used in the near future to improve bone health in children in order to prevent bone disease later in life. This approach emphasizes disease prevention early in life rather than disease treatment later in life, which is direction of the majority of research and care for osteoporosis.

This proposal for a bone health screening program would be a future research study that utilizes well known SNPs associated with variation in BMD, BMC, and bone geometry, as well as the genome-wide significant results of this current study. This proposed program would also act to inform public health practice and guidelines for management of children with genetic risk factors for poor bone health.

The goals of a bone health screening program include 1) identifying children at risk for poor bone health later in life, 2) begin interventions early in childhood to optimize peak BMD, and 3) provide skills and knowledge to high risk children in order to maintain healthy bones over time.

A calculated genetic risk score will be used to identify children with higher risk for poor bone health. This genetic risk score will take into account the number of SNPs (identified in previous literature as well as this study) associated with low BMD and BMC and poor bone geometry, sex, race, family history of bone disease, and age. A threshold will be established and

children with a genetic risk score above that threshold will be considered "high-risk" and will be recommended for further interventions. The approach of a genetic risk score was utilized in another recently published study[36]. This study used adult identified BMD-lowering variants and generated genetic risk scores to determine if previously identified variants in adults are associated with variation in BMD during childhood. A higher genetic risk score was found to be negatively linked with BMD and BMC at the age of 13 and was associated with a slower rate of bone accrual between the ages of 13 and 17 years[36]. While this study did not include sex or multiple site phenotypes, clinical validity was proven between a calculated genetic risk score and identifying individuals with poorer childhood bone health.

The genes and SNPs tested to determine a genetic risk score should include well-studied candidate genes and loci in adults as well as the genes and loci implicated in studies focused on children. Because the current research on candidate genes associated with bone health has been primarily focused on the elderly population and research on genes involved in bone development in children still needs further investigation, genes of interest will change as knowledge and understanding of genetic regulators of bone development increases as well as when we better understand the difference in genetic contributors to bone homeostasis at different periods of life. However, for the purpose of this pilot study, the panel of genes will stay the same; adjustments can be made in subsequent applications. Rapid changes in the genes on available panels is however a challenge for genetics in setting and is not something necessarily unique to bone genetics or this pilot study.

This proposal is for a future program, therefore there are significant limitations and assumptions being made currently. Assumptions that must be made in order for this program to be successful include:

1. The genes sequenced are significant and impactful in childhood bone health.
2. There are no other significant and impactful genes that affect childhood bone health.
3. A Next Gen Sequencing Panel (NGS) can be built to include well-known candidate genes and SNPs for bone health in adults and children.
4. Variations and mutations that are associated with lower peak bone mass are known and can be identified via NGS
5. The detection rate for these variations and mutations is over 90%.
6. The sensitivity, specificity, and positive predictive value for these variations and mutations are significant for poor bone health later in life.

It is clear given the above assumptions that need to be met that this program is for a future time. More research is needed to make this proposal a reality. However, given the significant public health burden of osteoporosis and the popularity of research on the genetics of bone health, this proposal may be a reality in the near future. If the above assumptions could be met and this screening program could be implemented, the associated interventions that would be recommended are outlined below.

The proposed pilot research study will take place in the city of Pittsburgh. The population will include five hundred healthy children; age 5 years that are seeing Pittsburgh primary care physicians (PCPs) for checkups and wellness visits. For the purpose of this proposal, the age of 5 years was decided because of the age of interest studied in the GWAS previously. Deciding on the age to target for a screening program proved to be a complicated matter. We want the program and interventions to begin early enough that skills are able to become habit, but also at an age when children will be compliant to diet and exercise changes.

Previous intervention studies have targeted children as young as seven years. Again, the decision to target children age 5 years is based on the population studied for our previous research, however, age may need to be adjusted in subsequent applications.

In order to reach this population, education of Pittsburgh PCPs on the genetic risk factors of poor bone health and the effectiveness of interventions during childhood will be needed. This educational plan for the local providers will include a baseline survey of current PCP knowledge on bone health as well as a continuing education online module for PCP's focused on the background issues of this program. Print material for all families meeting the above criteria will be created to give families information on 1) general strategies to maintain and optimize bone health during childhood and in adulthood, 2) genetic testing and associated benefits and risks, and 3) the recommended interventions of the program to optimize bone health. This print material will be provided to all local PCPs and will be handed out by the PCPs. Families that are interested in the screening program will schedule a pre-test counseling session with a genetic counselor prior to any genetic testing or the calculation of a genetic risk score. Children who have undergone genetic testing and are found to have a high genetic risk score will be recommended to participate in the interventions outlined below.

5.3 PROPOSED INTERVENTIONS

While a number of population based interventions have been implemented and studied to promote bone health, very few have been targeted at children, and even fewer have combined so many factors, including education, diet, and physical activity. Previous examples of interventions focused on bone health have also been established on a community level without any associated

screening program to identify those in the community at a higher risk based on race, genetic risk factors, or family history of bone disease. This proposed intervention intends to include as many of the factors that contribute to osteoporosis as possible in order to provide the most effective and all-encompassing prevention strategy.

Children identified as high-risk for bone disease later in life, through the above screening program, will be recommended to participate in this intervention program. Those that wish to participate will be instructed to undergo baseline BMD, BMC, and bone geometry measurements using DXA. Additionally a survey will be issued to the child's parents to assess the child's activity level, diet, and any exposures in the household. Following the baseline measurements, children in this program will be scheduled sessions to meet with a nutritionist and a physical trainer.

The first aspect of this intervention program is to ensure a healthy and sufficient diet rich in the nutrients need for healthy bones. As outlined in the Introduction chapter of this paper, vitamin D, calcium, vitamin K, potassium, and magnesium are necessary for strong bones. Additionally limiting salt intake, caffeine, and phosphate can promote favorable bone health[52]. In order to educate families and give the tools to promote and practice this healthy diet, this program intervention includes sessions with a nutritionist once a month for the first three months of the program and then once every four months following that.

Previous studies have demonstrated the effectiveness of dietary strategies on bone health. In one study, the DASH (Dietary Approaches to Stop Hypertension) diet, a calcium rich diet that is made up of mostly fruits, vegetables, and low fat dairy products, significantly reduced bone turnover, improved BMD, and improved calcium metabolism in adults[213]. Another study investigating the effects of a 30-month dietary intervention, combined supplements of dairy products fortified with calcium and vitamin D3 alongside nutrition and lifestyle counseling, on

BMD of postmenopausal women. The result of this study demonstrated that this dietary intervention significantly increased arm, spine, and total body BMD[214]. Based on the evidence of previous intervention programs, nutrition counseling is a key aspect of this program that will not only promote healthier bones but also have benefits towards children's overall health and hopefully have a positive impact on the family as a whole.

The second aspect of this intervention program is to increase the physical activity of children. Weight-bearing physical activity initiates bone remodeling and bone formation, making bones stronger as a result. Weight-bearing physical activity includes actions such as walking, running, jumping rope, playing tennis, hockey, and basketball, dancing, hiking, and lifting weights. It is recommended that children and adolescents should have sixty minutes of physical activity every day and that bone-strengthening activities should be done at least three days a week[215].

To maximize the benefit of physical activity on childhood bone health, this program will include group family physical training classes. These classes will allow for six family groups each, each group including any interested family member, and will occur once every two weeks for the first four months and then once per month for the following six months. The goals of these classes are to promote habitual physical activity, provide examples of bone-strengthening activities, and promote exercise as a family activity. At least one parent is required to be present with the child at each class and there is an expectation that the families will continue exercises in between classes and after the classes are finished.

Many previous studies have demonstrated the positive impact of exercise on bone health. One previous intervention program implemented in Sweden studied the influence of a three year school exercise program, in which children had physical education for 40 minutes each day, and results showed that this moderately intense exercise program in 7-9 year old children increased

bone mass, BMC, without increasing fracture risk[216]. Another study studied the relationship between habitual physical activity and bone geometry in premenarcheal girls. This study showed that physical activity affects bone health in a dose-depended manner. Girls that participated in a high level of physical activity had greater bone thickness, cross-sectional area, BMC, and BMD when compared to their physically inactive or moderate active counterparts. Additionally studies have shown that children and young adults who participate in intensive weight- bearing activity in elite sports have significantly greater BMD than less active controls and that the effects of activity on bone are site specific[59].

For this pilot study, we have elected not to include a control group to determine effectiveness of the interventions. Determining a suitable comparison group was not a straightforward issue. Several options of a control group included children determined to have a low genetic risk score that continue with the proposed interventions, children that are determined to have a low genetic risk score but are found to have poor bone health, through baseline measurements, that continue with the proposed interventions, or children determined to have a high genetic risk score that do not participate in the proposed interventions. Because each of these possibilities would be comparing the effectiveness of the intervention in a specific genetic risk score group or would be withholding beneficial interventions from those at high risk, rather than measuring the effectiveness of the interventions when children at risk are identified and participate in interventions early in life, we elected not to include a control group.

In order for this intervention program to be successful, several assumptions were made. These assumptions include:

1. These interventions will improve bone health outcomes of children with high genetic risk factors.

2. Skills and knowledge initiated at this age will be able to become habitual and implemented into life routine.
3. Guidelines have not yet been established for the recommendations of management of children found to be genetically predisposed to poor bone health.
4. This program would be a research study in order to inform public health practice and guidelines for management of at–risk children.
5. Families will comprehend the education provided in nutrition counseling and physical training sessions and will activity try to implement these skills into daily life.
6. Expected child bone growth curves are available for comparison.

5.4 INTERVENTION OUTCOMES AND EVALUATIONS

The projected outcomes of these interventions and the ways in which the impact of these interventions will be measured and evaluated are outlined in the Logic Model below (Figure 16). Short term goals are that 60% of families take the provided print materials with information on the bone health program, 30% of families schedule a genetic counseling session for pre-test counseling, and 10% of families decide to participate in the program within one year of the program's initiation. Short term outcomes will be evaluated by the uptake of print materials, the number of scheduled genetic counseling sessions, and the number of families that undergo baseline BMD measurements, genetic testing, and complete a lifestyle survey.

Program: Genetic Bone Health Screening and Intervention Program in Children

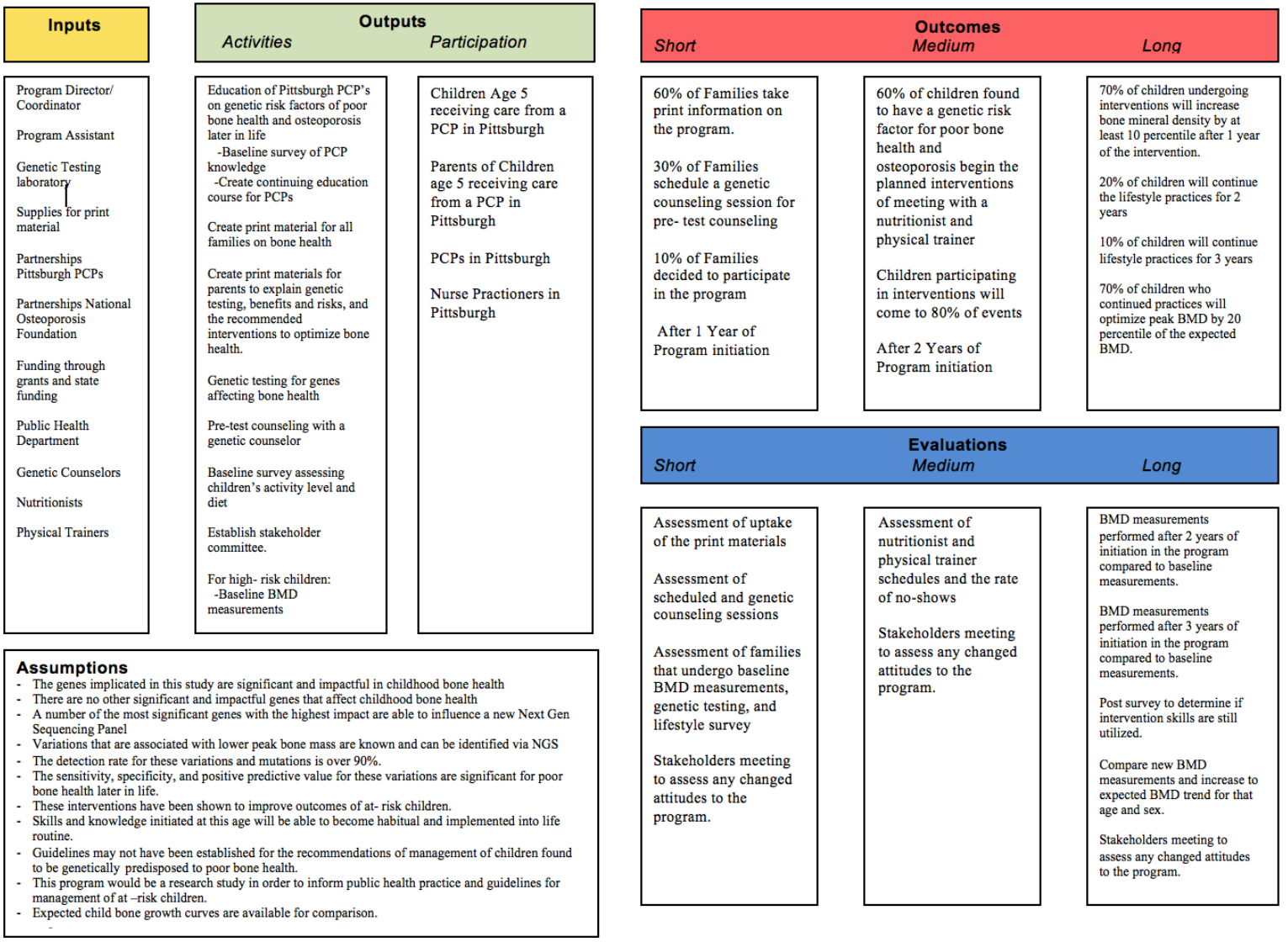

Figure 20: Logic Model

Medium-term goals are that 60% of children found to have a higher genetic risk score begin the planned interventions of meeting with a nutritionist and physical trainer, and that participating children will attend at least 80% of the program events. Reminder calls and messages can be used to remind families of program events. The medium-term goals are projected to be met within 2 years of the program's initiation. Medium-term outcomes will be evaluated through nutritionist and physical trainer schedules and the rate of no-shows or cancelations.

Long-term goals are that 70% of the children undergoing the recommended interventions will show at least a 10 percentile increase in bone mineral density after participating in the program for 1 year. Further, 20% of the children that began the program will continue to maintain healthy nutrition and physical activity promoted by the intervention. Ultimately, it is aimed that 70% of the children who continue the promoted lifestyle practices will optimize peak BMD by 20 percentile of their expected BMD. These goals are based on results of previous interventions and the increase seen in treatment groups. Long-term outcomes will be assessed through BMD measurements performed at two and three years following the initiation of the program in comparison to the baseline measurements. This data will also be assessed for trends or differences at different ages and for gender. Additionally, a post survey will be issued to determine if the intervention skills are still utilized. Lastly, a follow-up stakeholders meeting will be conducted to assess overall attitudes towards the program and benefits and challenges that were felt by the community.

A stakeholders meeting will also be planned following sort, medium, and long-term goals to assess any changed attitudes or concerns to the program and the impact on the community.

5.5 ROLES OF A GENETIC COUNSELOR

As the genetic field continues to learn more about the genetic contributions of multifactorial traits and diseases, the roles of genetic counselors must adapt to meet families' concerns, address trends in direct-to-consumer testing, and be able to speak to recurrence risk of complex disorders. For the purpose of genetic screening to identify children with a high risk for bone disease later in life, there are three main skills that must be slightly adapted to meet the unique needs of such a program; these skills include disease education, preservation of patient autonomy, and informed consent.

Multifactorial disease education is an essential skill for genetic counselors in any setting; however, education is largely done on well-known genetic conditions in which the cause, risks, medical management, and familial implications are well studied. The genetics of bone development and the genetic contribution to osteoporosis is lacking in each of the above areas; the interactions of genetics and environment are still largely unknown, the predictive value of genetic risk factors is not known, medical management for children with a genetic predisposition is not established, and we cannot speak to familial risks of osteoporosis or the patterns of inheritance of genetic variants. Counselors should be transparent about the many areas of bone biology that are not clear during pre-test counseling. There should be a clear and understandable illustration of multifactorial inheritance for patients to understand that genetic risk factors and lifestyle factors are both important when discussing risk of osteoporosis. Pre-testing counseling is also a time in which better bone health practices for all members of the family, such as exercise and nutrition, can be shared. While osteoporosis is not often thought to be a disease explained by genetic counselors, counselors have the skill and expertise to clearly explain the genetic contributors and environmental factors that pose a risk to patients.

Because this program would act as a research study with fairly extensive commitments including x-rays, genetic testing, and sessions with several specialists, participant autonomy must be stressed. Parents have the ability to decide to opt-in for genetic test and have the ability to opt-out at any time during the screening or intervention program. Again, the training of genetic counselors prepares them to consent, with a nondirective nature, families into studies and clearly explain the benefits and risks that should be considered.

Lastly, informed consent is required for any patient pursuing genetic testing or participating in a study. This particular screening program does involve some important risks and considerations that parents should be aware of before deciding to pursue genetic testing, even if for variants that affect bone health. First, this screening program is technically genetic testing of minors for an adult onset disease. Genetic testing of minors for adult onset disorders is not recommended until the child can make their own decisions, unless there would be a change in medical management. While there is no treatment that would be initiated based on the genetic testing results, interventions that have been proven to improve bone health before peak bone mass can have significant impact on bone health later in life, improving the overall health and prognosis later in life. Knowing if there is a genetic predisposition to bone disease is only helpful if appropriate interventions are utilized. Another important thought that needs to be considered with families interested in the screening program is the potential familial implications. A high genetic risk score may suggest that other family members also have a genetic predisposition to osteoporosis. While this could mean other family members, particularly older individuals, are evaluated earlier for poor bone health, interventions for older family members may not be as effective as when the interventions are implemented during childhood. However, a low genetic risk score does not mean that there is no risk of osteoporosis and strategies can still be implemented to maintain bone health. These are

several important issues to a bone health genetic screening program that must be conveyed to families before they participate in the program, and a critical role of a participating genetic counselor is the ability to explain these issues to families and ensure informed consent is being prioritized.

In summary, osteoporosis represents a major public health concern, and while efforts are currently more focused on treatment of the disease later in life, prevention strategies should begin during childhood in order to maximize peak bone mass. Individual variation in bone health and peak bone mass is largely controlled by genetics in addition to environmental and behavioral factors. If the genetic variants that are associated with poor bone health, there is a possibility of initiating a public health genetic screening program aimed at identifying children with genetic predispositions to osteoporosis later in life. Identifying these children will allow us to implement effective interventions, such as promoting healthy nutrition and physical activity, early, optimizing the benefit while bone mass is still being formed and developed. Ultimately, the combination of a genetic screening program and corresponding intervention program aims to utilize the genetics of bone disease in osteoporosis prevention.

APPENDIX A: GENES NEIGHBORING GWAS SIGNIFICANT LOCI

A.1.1 Hip BMD

HKDC1, HK1, TACR2, TSPAN15, NEUROG3, C10ORF35, COL13A1

A.1.2 Hip BMC

OLFM3, DNAJA1P5, COL11A1

A.1.3 Femoral Neck CSA

TRAT1, GUCA1C, MORC1, FLJ22763, LINC00488, DPPA2, DPPA4, FLJ25363

A.1.4 Femoral Neck Section Modulus

Chromosome 2:

KIAA1715, EVX2, HOXD13, HOXD12, HOXD11, HOXD10, HOXD9, HOXD8, HOXD-AS2, MIR10B, HOXD4, HOXD3, HOXD-AS1

Chromosome 12:

NAV3

APPENDIX B: LOCUSZOOM PLOTS OF SUGGESTIVE LOCI

B.1.1 Hip BMD

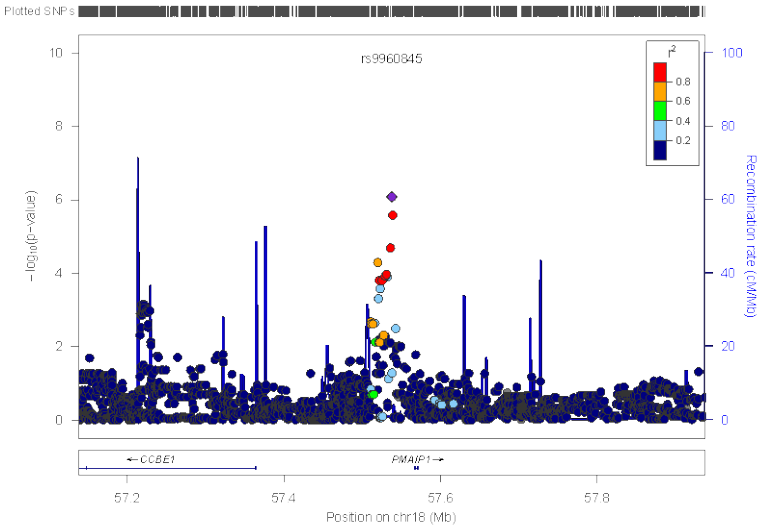

Figure 21: LocusZoom Plot for Hip BMD Suggestive Locus #1

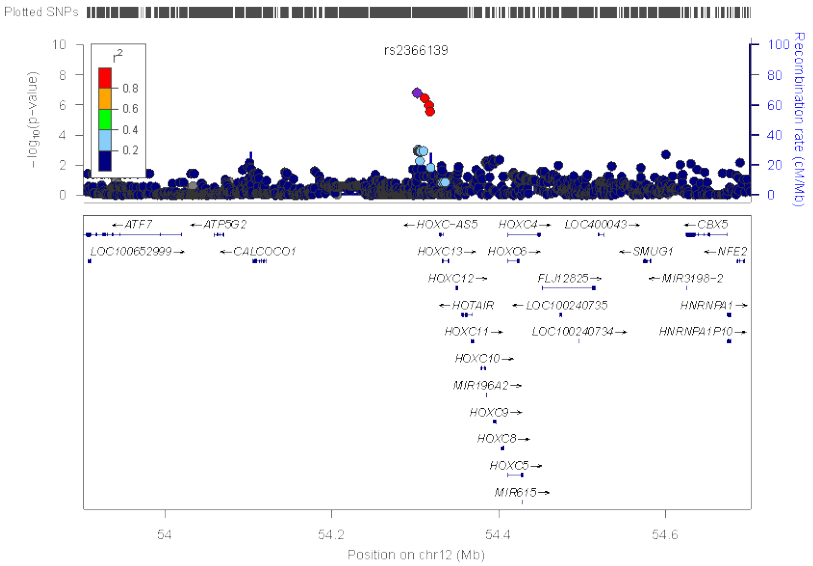

Figure 22: LocusZoom Plot for Hip BMD Suggestive Locus #2

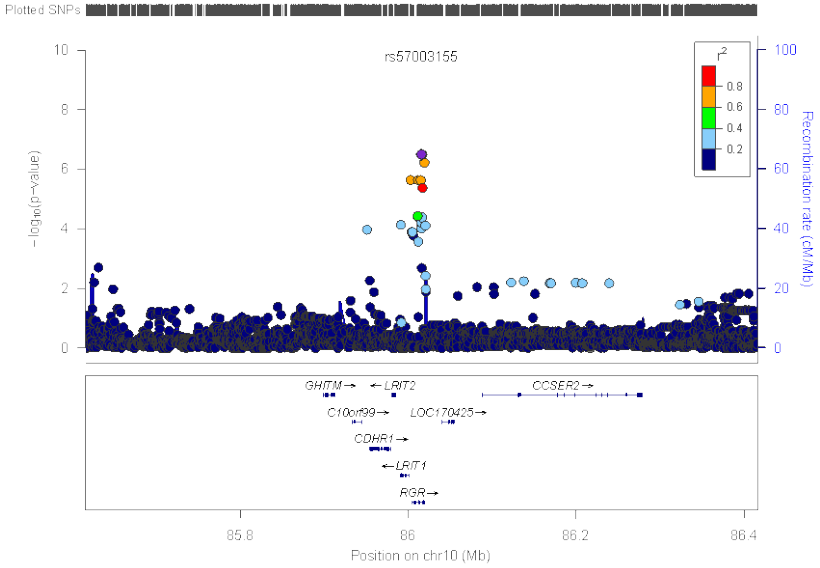

Figure 23: LocusZoom Plot for Hip BMD Suggestive Locus #3

B.1.2 Hip BMC

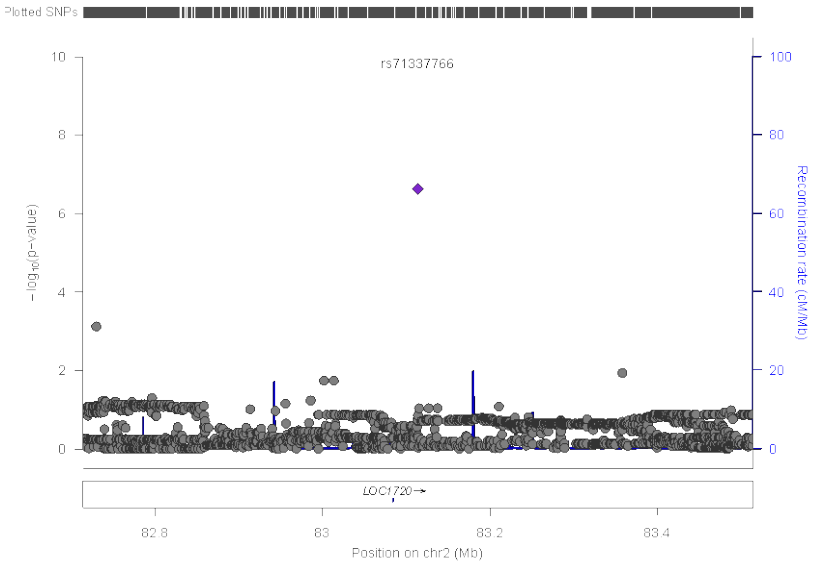

Figure 24: LocusZoom Plot for Hip BMC Suggestive Locus #1

B.1.3 Spine BMC

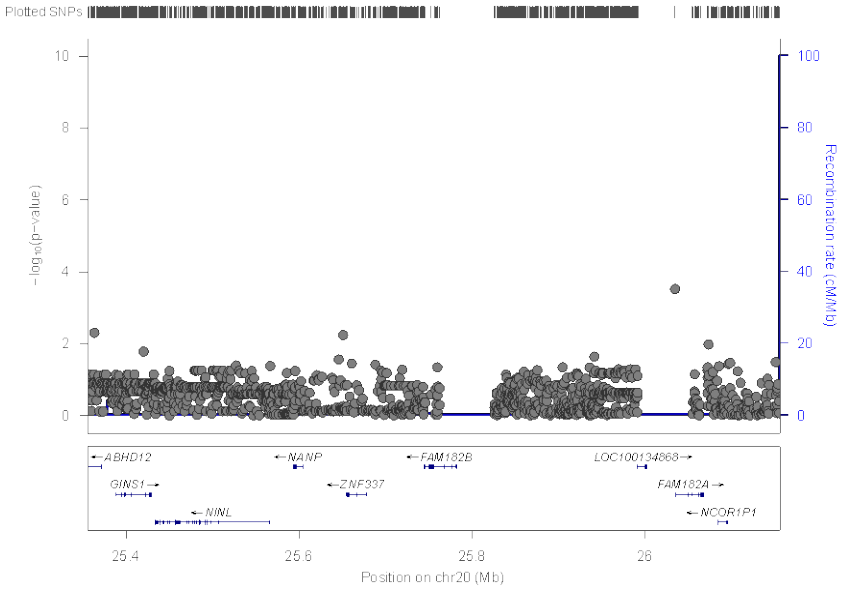

Figure 25: LocusZoom Plot for Spine BMC Suggestive Locus #1

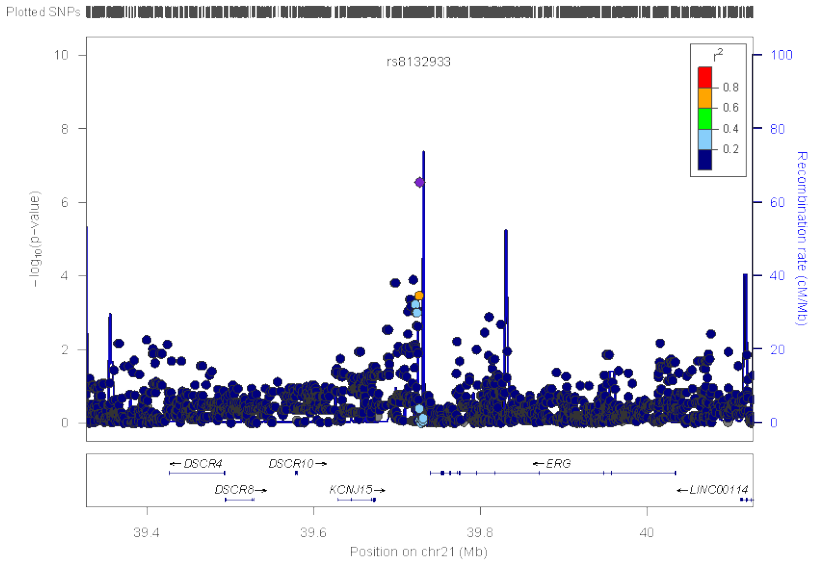

Figure 26: LocusZoom Plot for Spine BMC Suggestive Locus #2

B.1.4 Head BMC

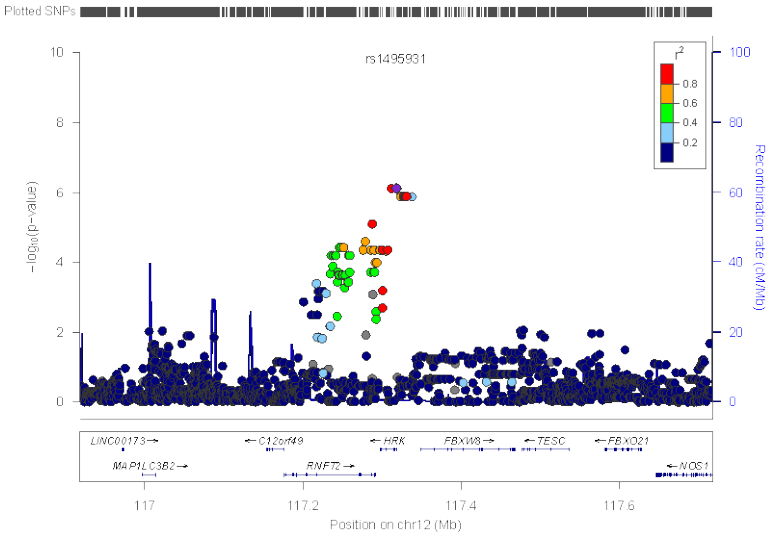

Figure 27: LocusZoom Plot for Head BMC Suggestive Locus #1

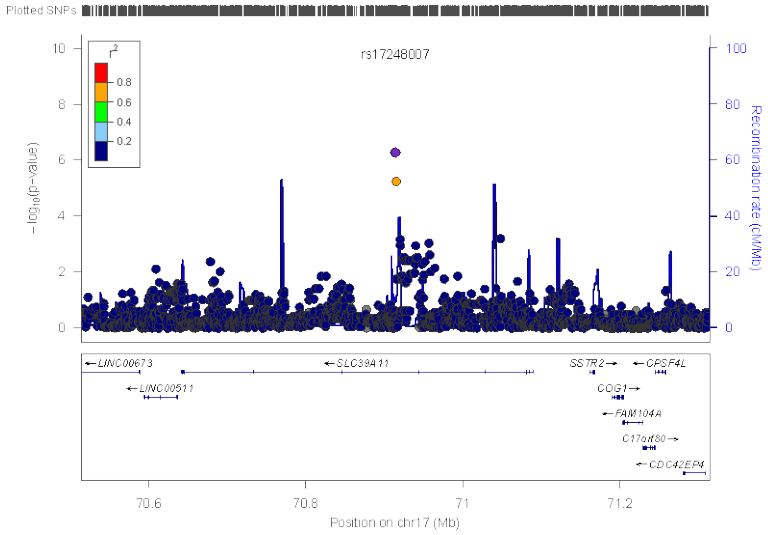

Figure 28: LocusZoom Plot for Head BMC Suggestive Locus #2

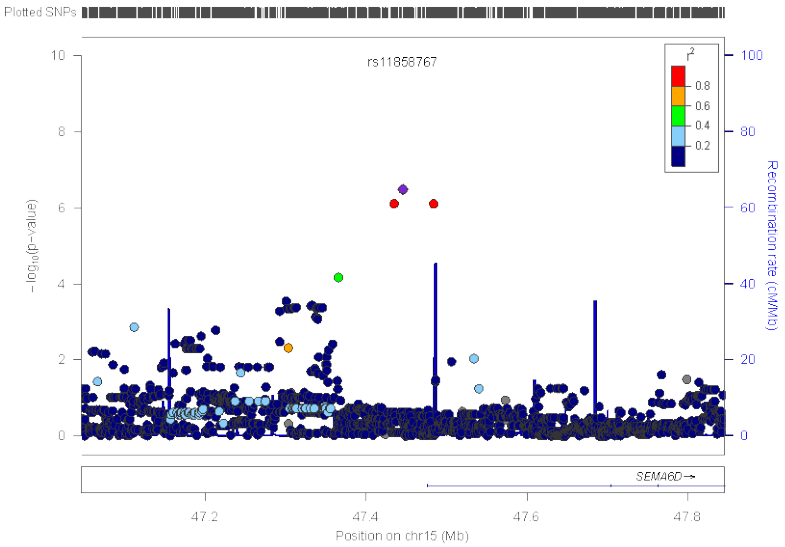

Figure 29: LocusZoom Plot for Head BMC Suggestive Locus #3

B.1.5 Whole Body BMC (Excluding Head)

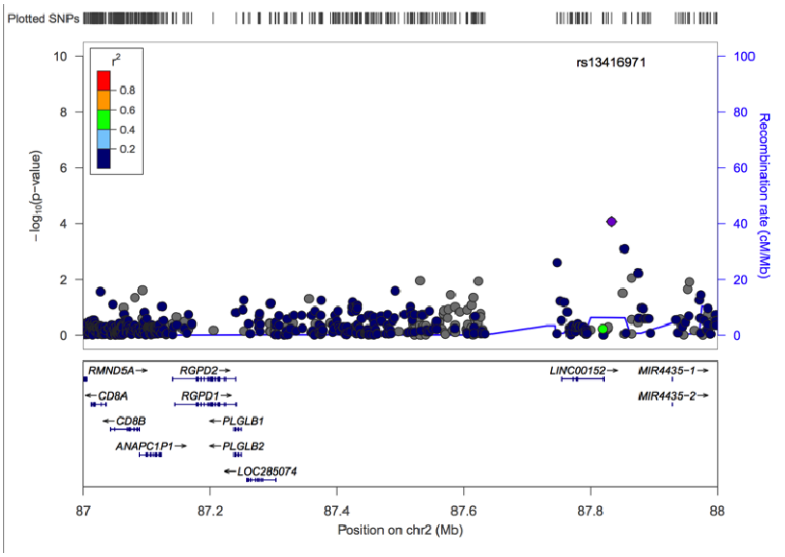

Figure 30: LocusZoom Plot for Whole Body BMC (Excluding Head) Suggestive Locus #1

B.1.6 Femoral Neck CSA

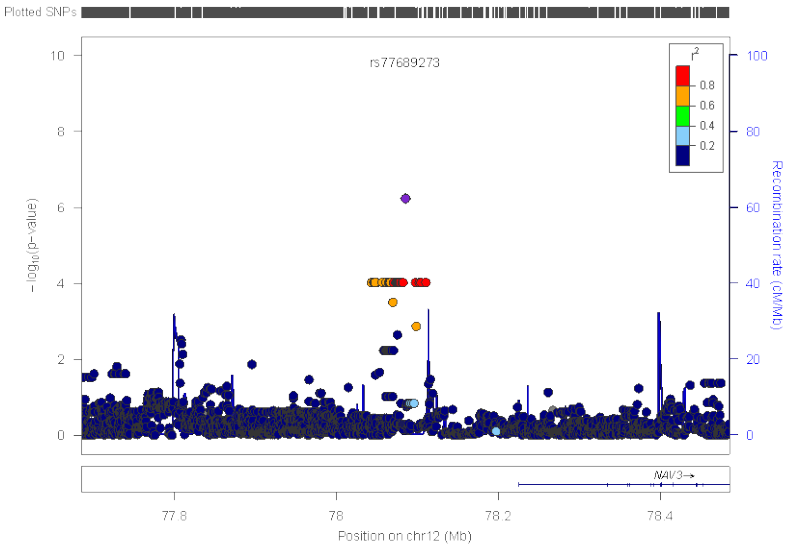

Figure 31: LocusZoom Plot for Femoral Neck CSA Suggestive Locus #1

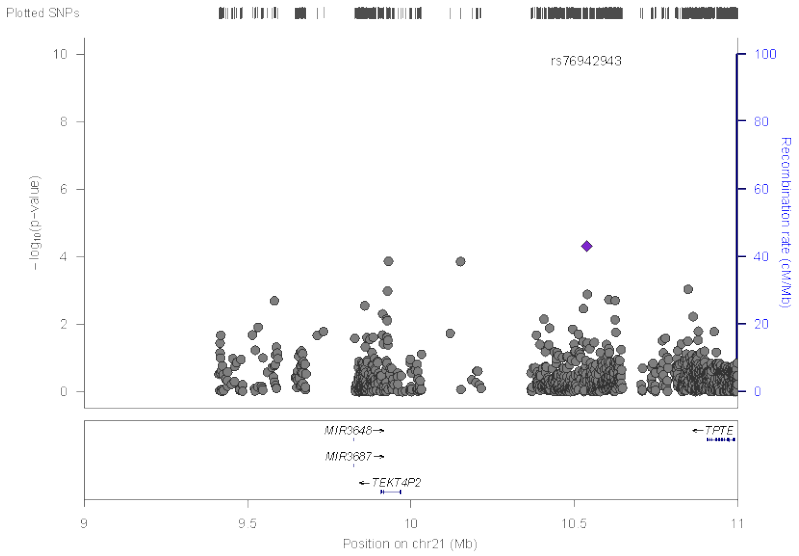

Figure 32: LocusZoom Plot for Femoral Neck CSA Suggestive Loci #2

B.1.7 Femoral Neck Section Modulus

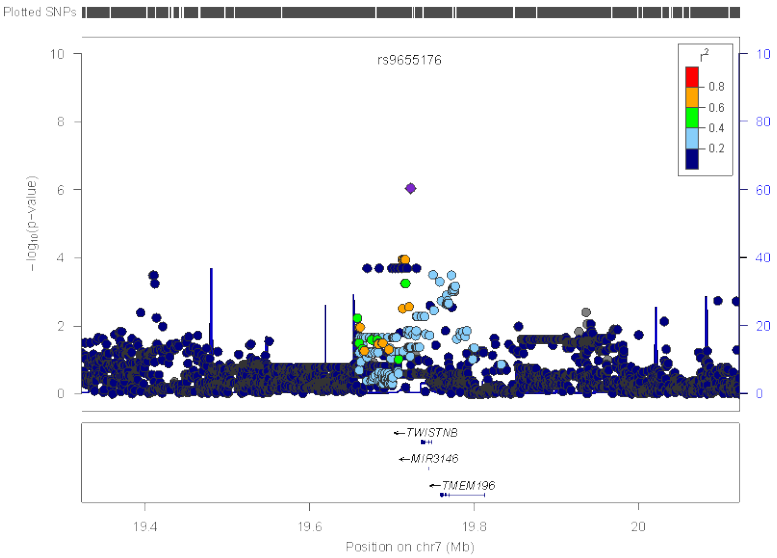

Figure 33: LocusZoom Plot for Femoral Neck Section Modulus Suggestive Locus #1

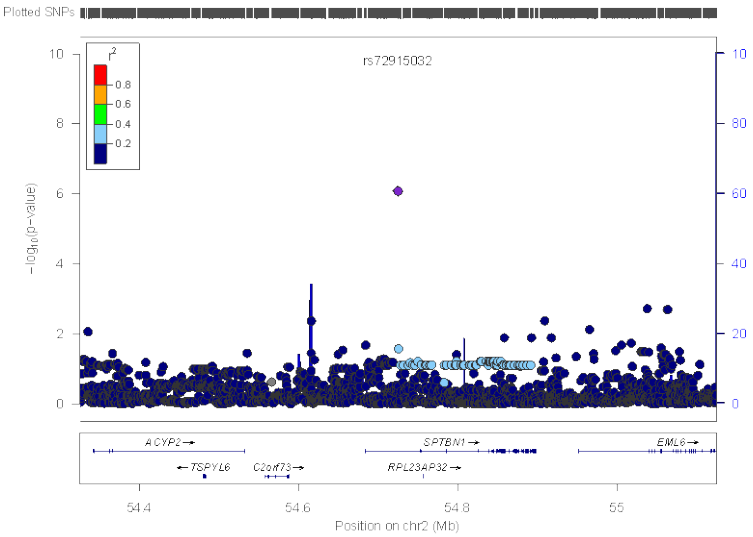

Figure 34: LocusZoom Plot for Femoral Neck Section Modulus Suggestive Locus #2

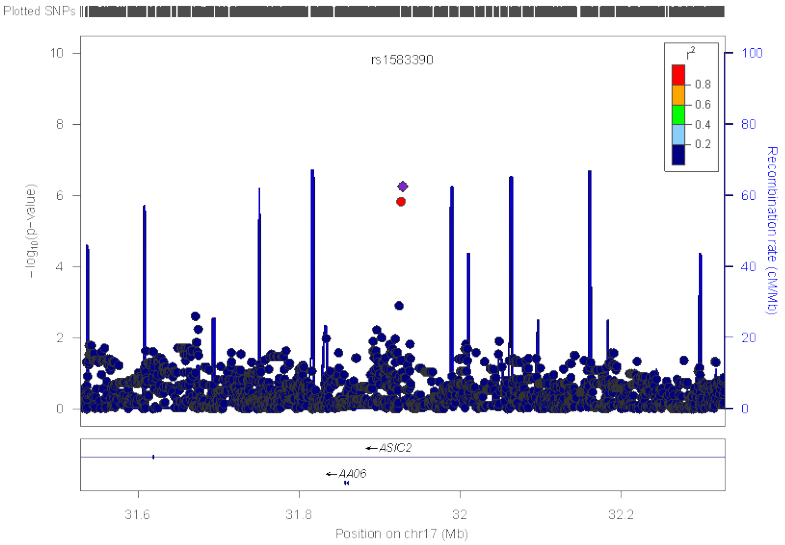

Figure 35: LocusZoom Plot for Femoral Neck Section Modulus Suggestive Locus #3

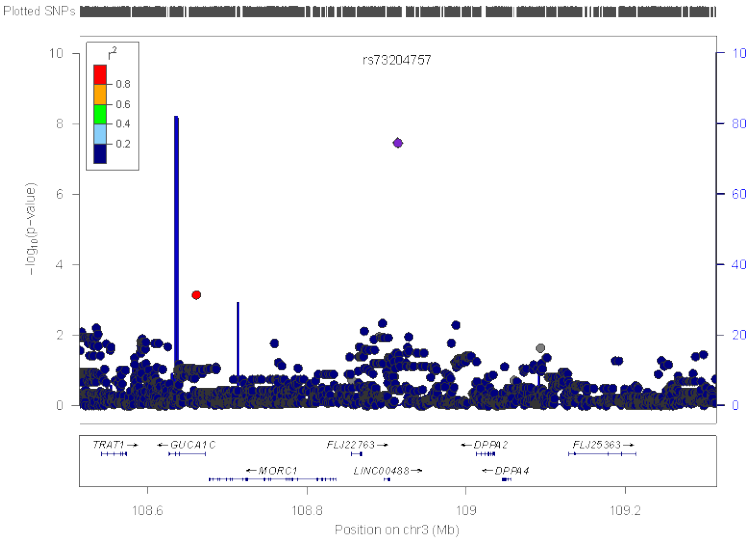

Figure 36: LocusZoom Plot for Femoral Neck Section Modulus Suggestive Locus #4

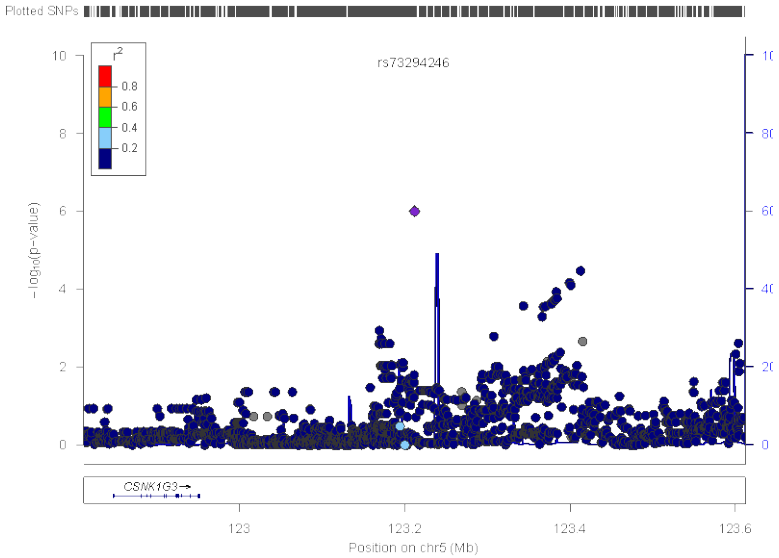

Figure 37: LocusZoom Plot for Femoral Neck Section Modulus Suggestive Locus #5

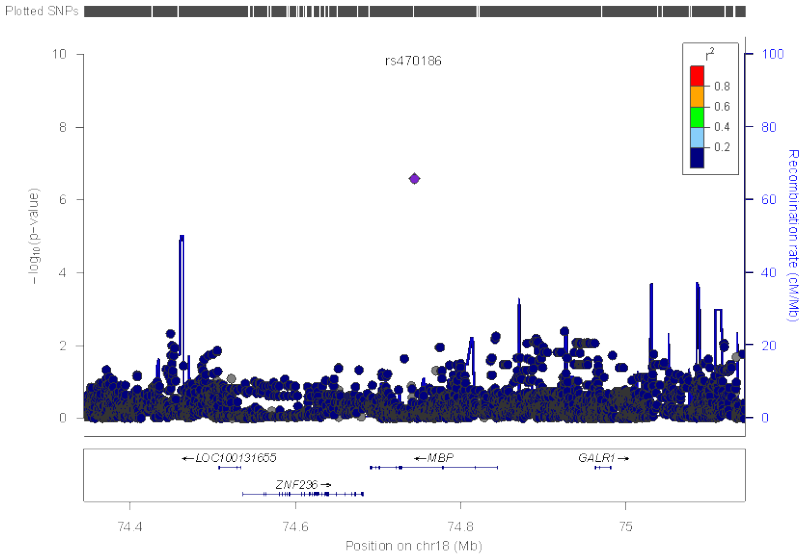

Figure 38: LocusZoom Plot for Femoral Neck Section Modulus Suggestive Locus #6

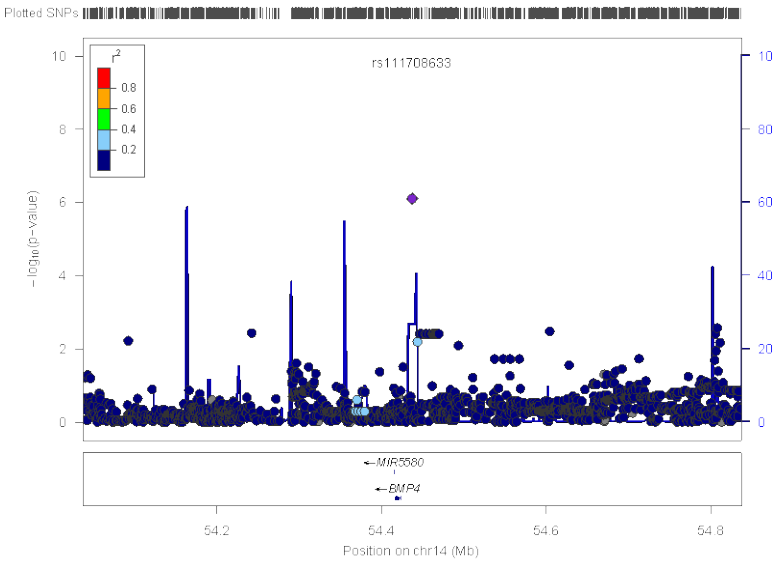

Figure 39: LocusZoom Plot for Femoral Neck Section Modulus Suggestive Locus #7

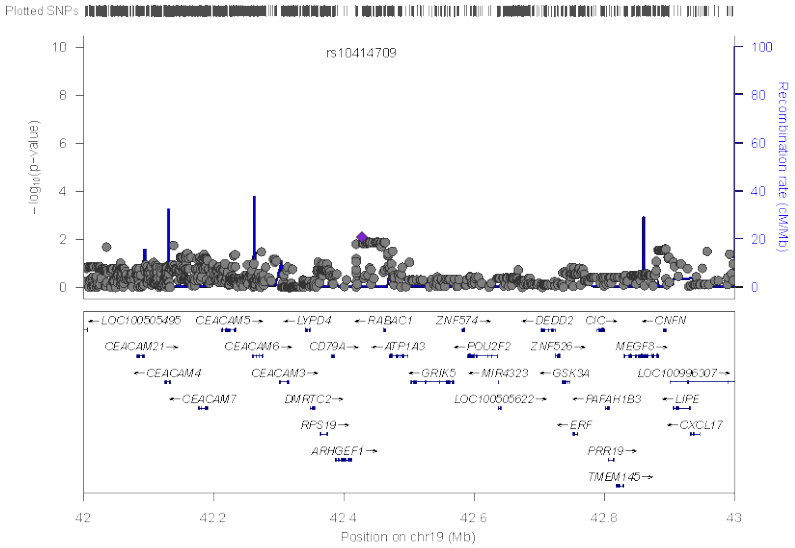

Figure 40: LocusZoom Plot for Femoral Neck Section Modulus Suggestive Locus #8

B.1.8 Femoral Neck Width

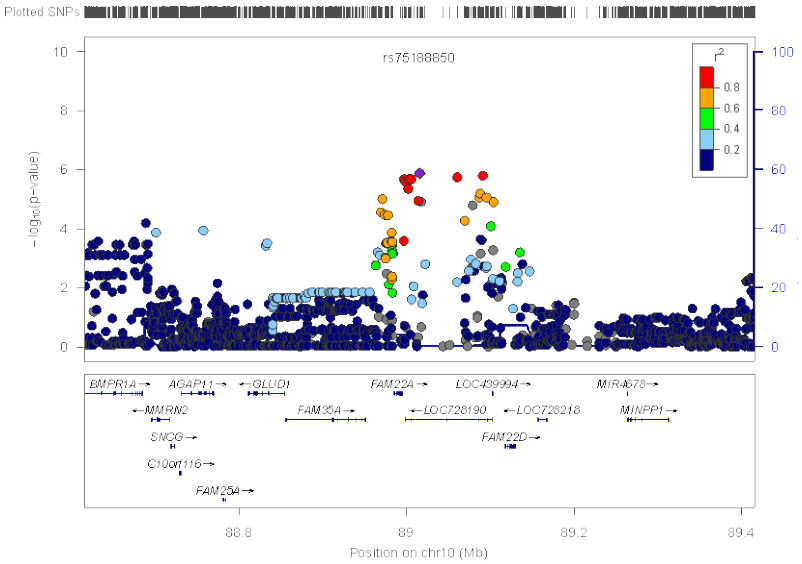

Figure 41: LocusZoom Plot for Femoral Neck Width Suggestive Locus #1

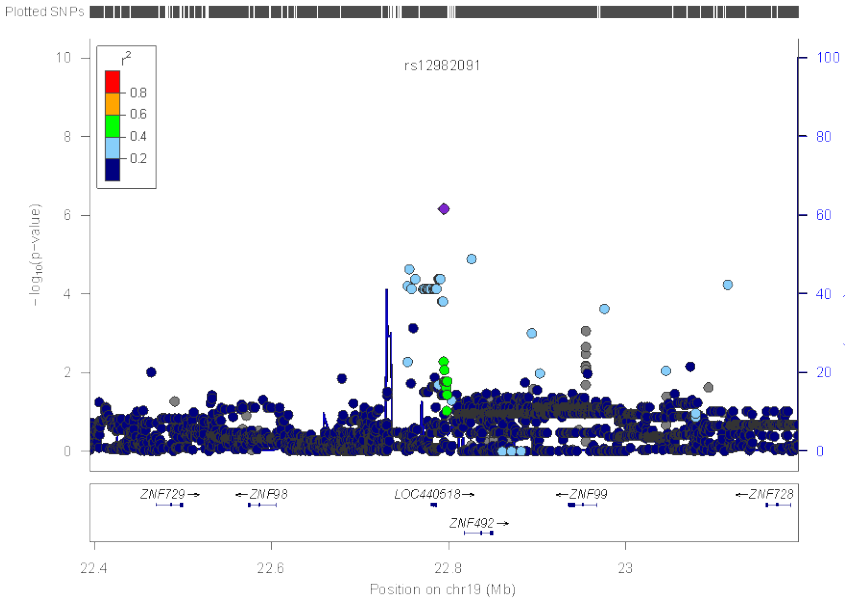

Figure 42: LocusZoom Plot for Femoral Neck Width Suggestive Locus #2

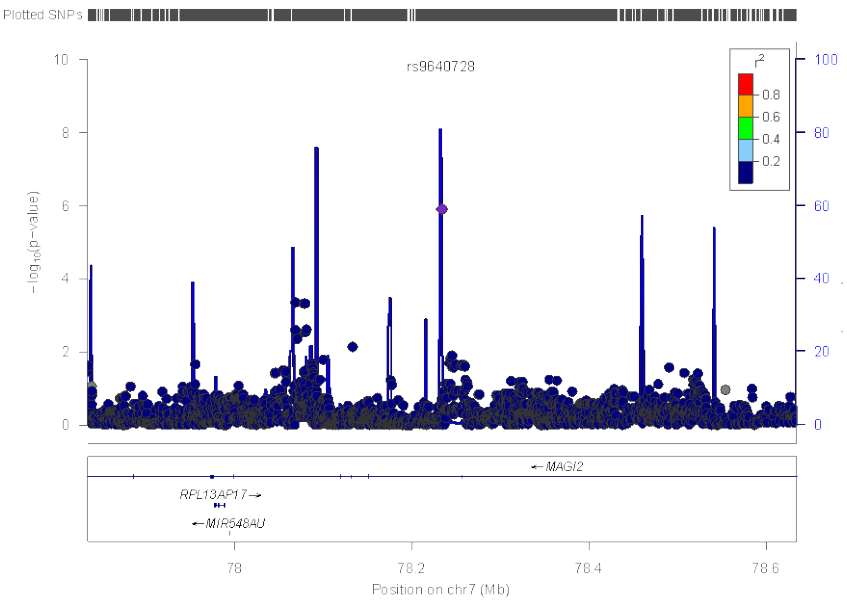

Figure 43: LocusZoom Plot for Femoral Neck Width Suggestive Locus #3

APPENDIX C: QQ PLOTS FOR BONE PHENOTYPES

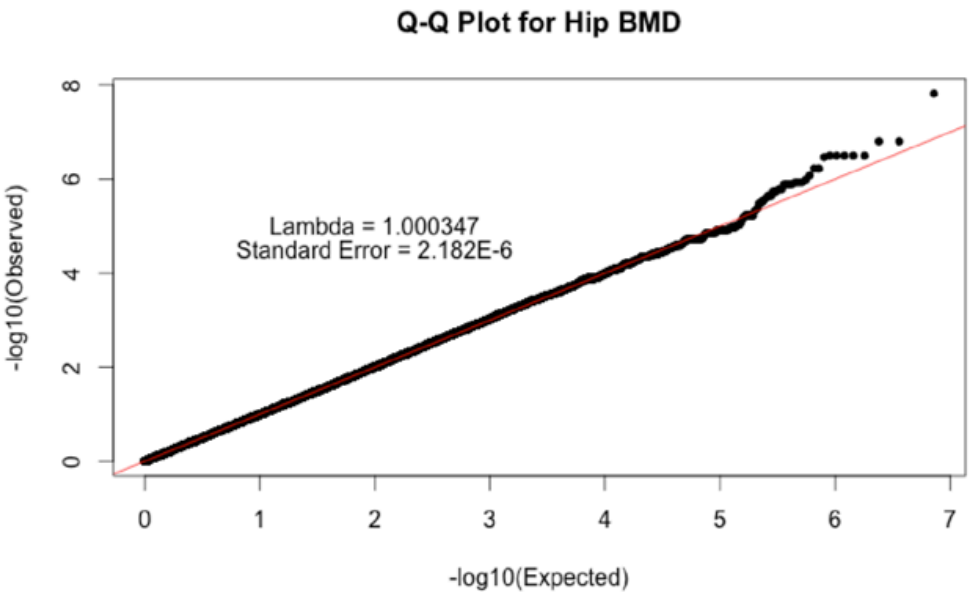

Figure 44: QQ Plot for Hip BMD

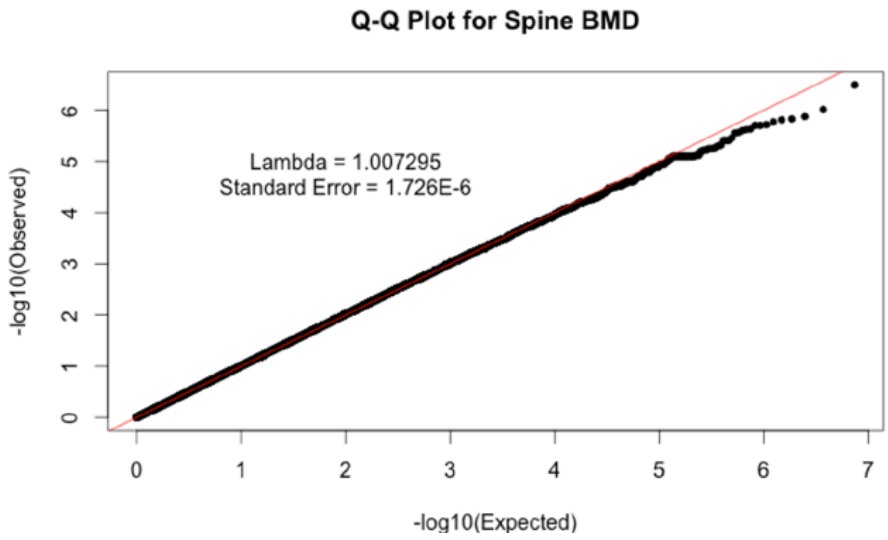

Figure 45: QQ Plot for Spine BMD

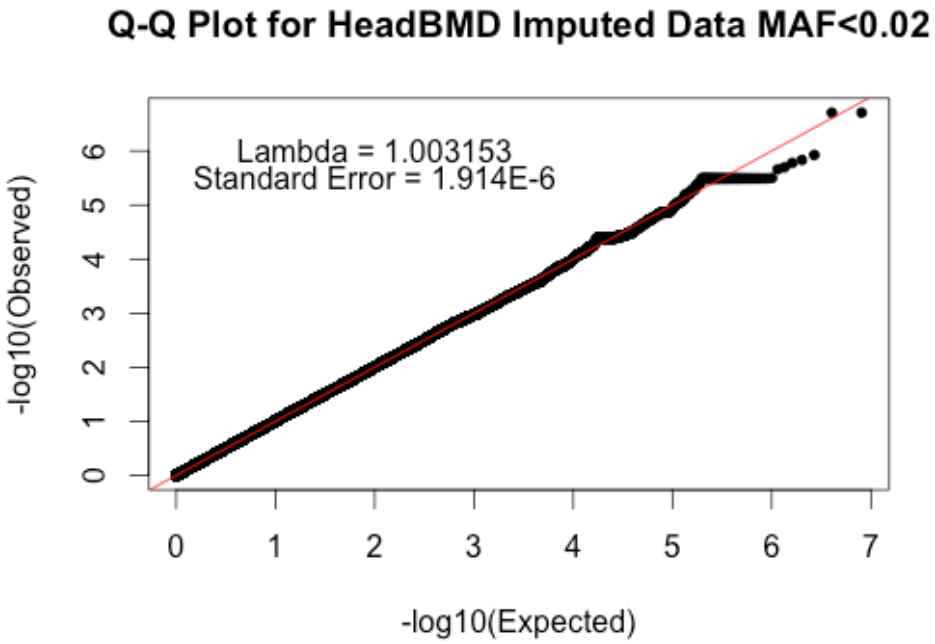

Figure 46: QQ Plot for Head BMD

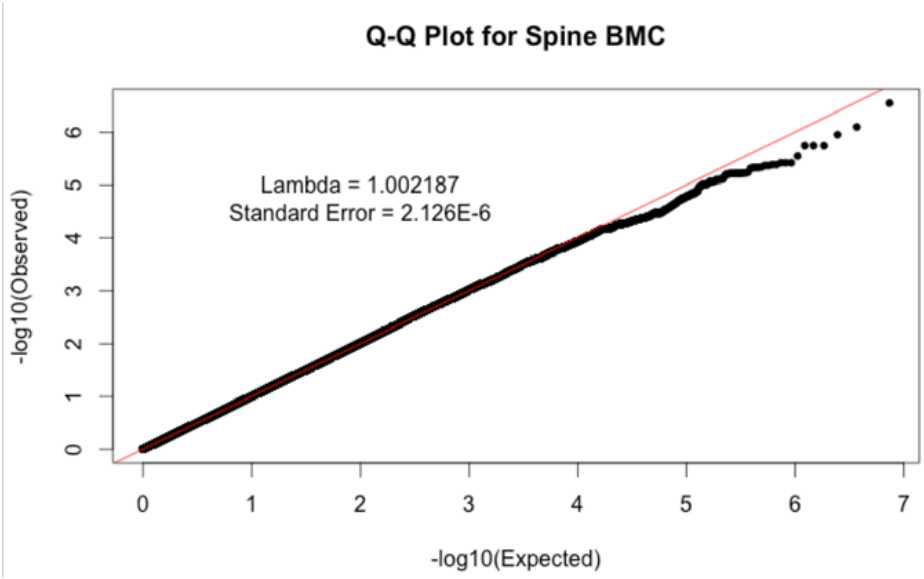

Figure 47: QQ Plot for Spine BMC

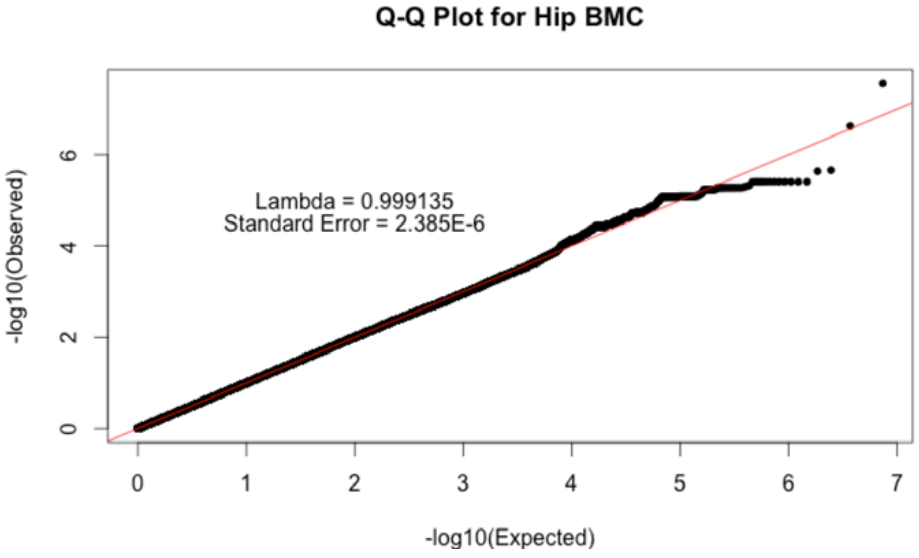

Figure 48: QQ Plot for Hip BMC

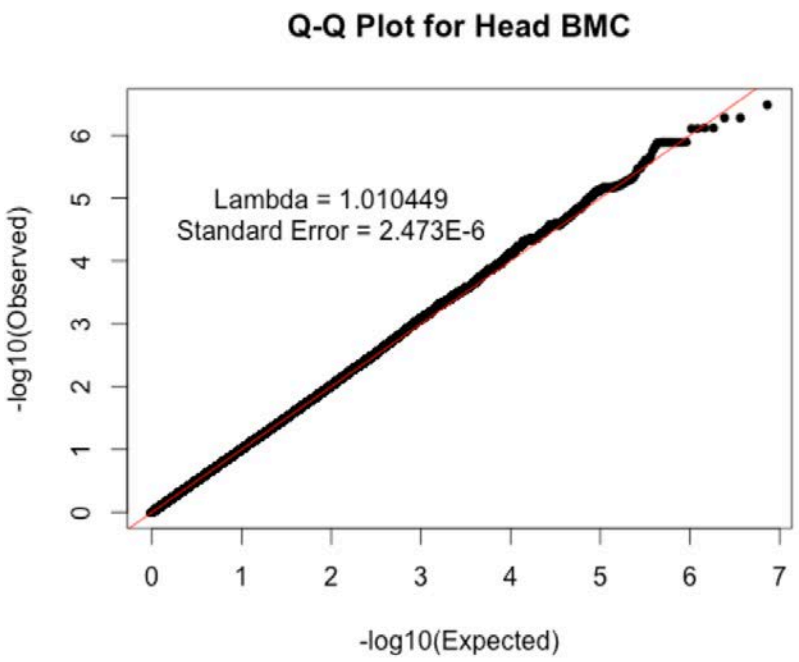

Figure 49: QQ Plot for Head BMC

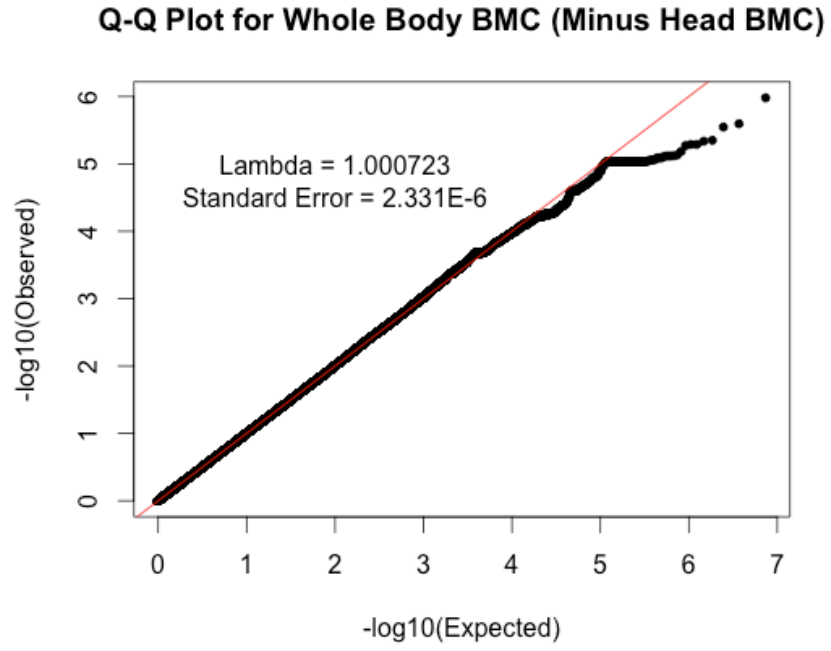

Figure 50: QQ Plot for Whole Body BMC (Excluding Head)

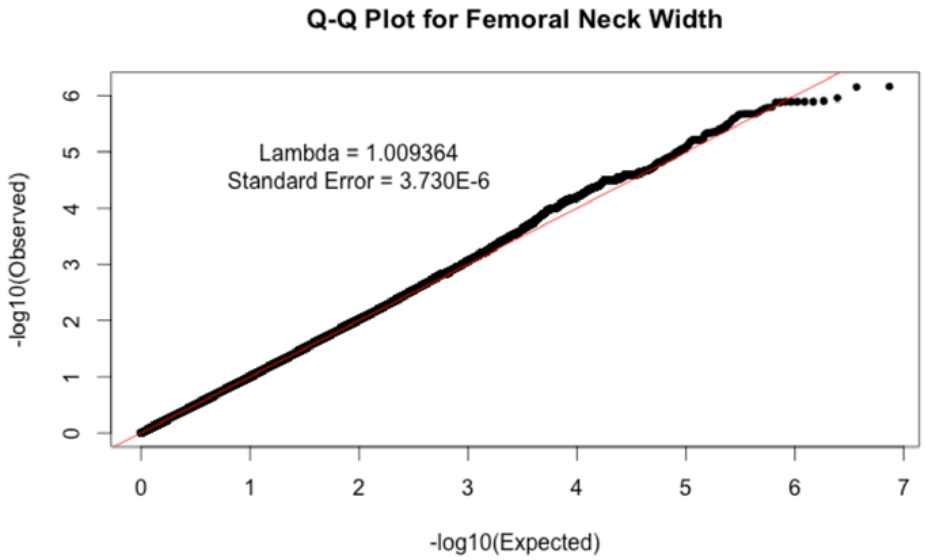

Figure 51: QQ Plot for Femoral Neck Width

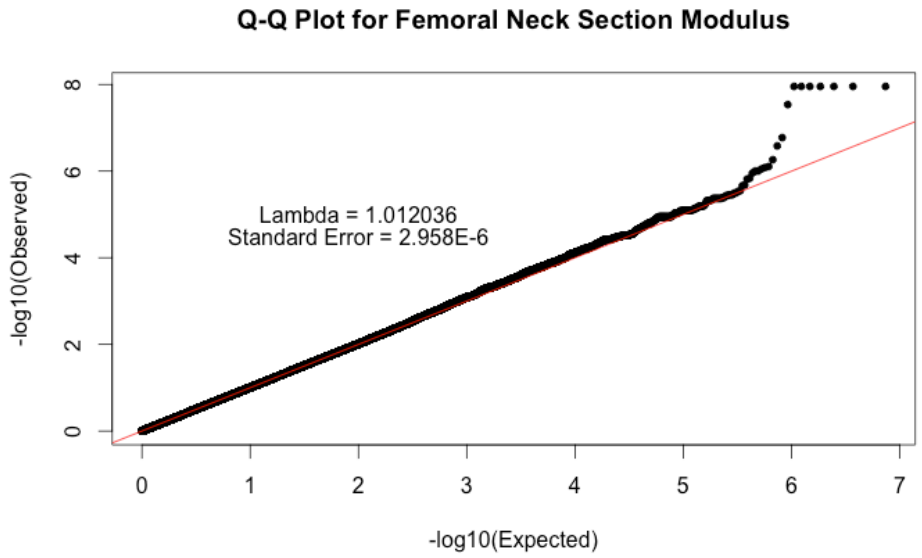

Figure 52: QQ Plot for Femoral Neck Section Modulus

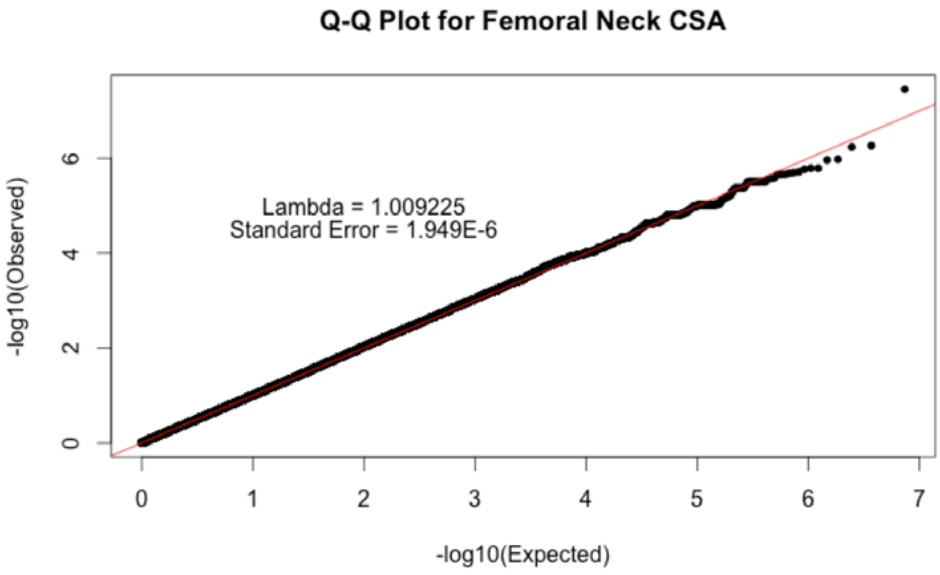

Figure 53: QQ Plot for Femoral Neck CSA

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