The genetics of osteoporosis

Thesis

UNRESTRICTED

THE GENETICS OF OSTEOPOROSIS

Emma Duncan

The Open University

Ph.D. thesis

2001

DATE OF SUBMISSION: 25 JUNE 2001
DATE OF AWARD: 19 FEBRUARY 2002

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Abstract

This thesis presents epidemiological and gene mapping studies of the genetics of osteoporosis and low bone mineral density (BMD). BMD is a highly heritable trait. However the genes that underlie the population variance in BMD remain unknown.

Genetic epidemiological studies of families collected for gene mapping investigations demonstrated significantly low BMD in siblings and relatives of probands with osteoporosis. A sibling recurrence risk ratio (λ_S) for low BMD was established, with λ_S of 6.26 at lumbar spine (LS) and 5.24 at femoral neck (FN), and heritability of BMD estimated at 60% for LS and 48% for FN. There was also evidence of both site- and gender-specific genetic effects.

A large candidate gene linkage study demonstrated linkage of BMD with several loci, notably Parathyroid Hormone Receptor Type 1 (PTHR1), type 1 Collagen alpha-1 (COL1A1), type 2 Collagen alpha-1/Vitamin D Receptor, Interleukins 1,4 and 6, Epidermal Growth Factor, RANKL and Estrogen Receptor-alpha.

Mutation screening of PTHR1 exons and promoter regions revealed both previously described and new polymorphisms. Association of BMD at LS with a polymorphism present in exon M7 of PTHR1 was demonstrated in both population-based and within-family association studies.

A within-family association study of a polymorphism in the first intron of COL1A1 found no evidence of association with BMD. However, when only maternal

transmissions were considered, there was evidence of association with BMD at FN, suggesting the possibility of imprinting of this gene.

Further genetic studies of PTHR1 and other genes identified as contributing to the population variance of BMD will help clarify their roles in the determination of BMD and the development of osteoporosis.

Certificate of Originality

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma of a university or other institute of higher learning, except where due acknowledgement is made in the text.

I also declare that the intellectual content of this thesis is the product of my own work, even though I may have received assistance from others on style, presentation and language expression.

Signed

Emma Duncan

Acknowledgements

I would like to acknowledge the collaboration and help received during the course of this project.

Chapter 3: Genetic Epidemiology of Osteoporosis and Bone Mineral Density

Family recruitment, particularly through female probands, was aided by research nurses Bente Jakobsen, Kate Dymott, Rebecca Sharp, and Alison James (Department of Endocrinology and Metabolism, Nuffield Orthopaedic Centre, Oxford, UK).

The Bone Densitometry Unit at the Nuffield Orthopaedic Centre generously gave participants in this project free access to bone densitometry.

Prof Janet Sinsheimer (Department of Biomathematics, University of California, Los Angeles, USA, formerly of the Wellcome Trust Centre for Human Genetics, Oxford, UK) and Dr Lon Cardon (Wellcome Trust Centre for Human Genetics) were the source of useful discussions and statistical assistance.

Chapter 4: A Candidate Gene Linkage Study in Families with Osteoporosis

I would like to thank Prof Paul Wordsworth who generously allowed me use of bench space in his laboratory at the Wellcome Trust Centre for Human Genetics. This incorporated not only the physical space but also use of a computer, refrigeration and freezer facilities, sharing of group resources including computer programmes, inclusion in his laboratory's journal club as well as his enthusiastic support of the work.

Technical assistance was received from the core staff of the Wellcome Trust Centre for Human Genetics, who prepared 6% polyacrylamide gels for genotyping. Technical assistance was also received from Dr Matthew Brown (Wellcome Trust Centre for Human Genetics).

Statistical analysis was assisted by Dr Matthew Brown, with much useful discussion with Prof Janet Sinsheimer, Prof Kenneth Lange (Department of Biomathematics, University of California, Los Angeles, USA), Prof Chris Amos (University of Texas, Houston, Texas, USA) and Prof John Eisman (The Garven Institute, Sydney, Australia).

Chapter 5: The Parathyroid Hormone Receptor Type 1: High Density Linkage Mapping and Mutation Screening

Denaturing high performance liquid chromatography using the Wave® machine was performed by Mr Lorne Lonie, Wellcome Trust Centre for Human Genetics. 40% acrylamide gels for sequencing were prepared by the core staff of the Wellcome Trust Centre for Human Genetics.

Chapter 6: Association Studies of the COL1A1 Sp1 Binding Site Polymorphism

Technical assistance with genotyping was provided by Mr James Colley, research assistant at Oxagen Ltd, Abingdon, Oxfordshire, UK.

Chapter 7: Association Studies of PTHR1

I would like to thank Dr Andre Uitterlinden who generously allowed me to work in his laboratory at Erasmus University, Rotterdam. Mr Pascal Arp provided technical

assistance in the Netherlands. Mr Andrew Timms, Wellcome Trust Centre for Human Genetics, provided technical assistance in the UK. Statistical assistance was received from Dr Matthew Brown.

I would like to thank both my supervisors, Prof John Wass (Department of Endocrinology and Metabolism, Nuffield Orthopaedic Centre, Oxford, UK) and Prof Andrew Carr (Nuffield Department of Surgery, University of Oxford, Oxford, UK) for their considerable help and sensible advice with this project. In particular, I would like to thank Prof Wass for his unstinting enthusiasm, fellowship and support.

I would also like to thank the charity Action Research, who awarded me a research fellowship and were the sole source of funding for this project.

All students undertaking a PhD demand a lot from their families, who give their love and support without counting the cost. I would like to thank my husband, Matthew, for his encouragement and delight in this work. My son Rory, who was born during the project, has been a daily source of joy: his photograph by the computer has encouraged me to finish writing the thesis.

For Matthew and Rory.

Table of Contents

Abbreviations

Å	Ångstrom
AA	amino acid
ANOVA	analysis of variance
AP-1	Activating Protein-1 (a transcription factor)
BMD	bone mineral density
BMI	body mass index
bp	base pair
BSA	bovine serum albumin
BUA	broadband ultrasound attenuation
Cbfa-1	core binding factor a1
CI	confidence interval
cM	centiMorgan
CSF-1	colony stimulating factor 1
C-terminal	carboxy-terminal
ddNTP	dideoxynucleotides
DXA	dual energy X-ray absorptiometry
d.f.	degrees of freedom
DHPLC	denaturing high performance liquid chromatography
dNTP	deoxynucleotides
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
ER-α	Estrogen receptor-alpha
IBD	identical by descent
IBS	identical by state
IGF	insulin-like growth factor
IL	interleukin
IP_3	inositol tris-phosphate
kb	kilobase
kDa	kiloDalton

λ_S	sibling recurrence risk ratio
LOD	logarithm of odds
MANOVA	multiple analyses of variance
Mb	Megabase
MLS	maximum LOD score
MW	molecular weight
N-terminal	amino-terminal
OPG	osteoprotegerin
PAP	pedigree analysis package
PCR	polymerase chain reaction
PIC	polymorphism information content
PTH	parathyroid hormone
PTHR1	parathyroid hormone receptor type 1
PTHrP	parathyroid hormone-related peptide, also known as PTH-like peptide
QCT	quantitative computerised tomography
QTDT	quantitative transmission disequilibrium testing
QUS	quantitative ultrasound
RANK	receptor activator of NF-κB
RANKL	receptor activator of NF-κB ligand
RFLP	restriction fragment length polymorphism
s.e.	standard error
SD	standard deviation
SDS	sodium dodecyl sulfate, also known as lauryl sulfate
SDW	sterile distilled water
SNP	single nucleotide polymorphism
SOLAR	sequential oligogenic linkage analysis routines
SOS	speed of sound
TBE	Tris-borate EDTA
TDT	transmission disequilibrium testing
TE8	Tris-EDTA buffer pH 8
TGF-β	transforming growth factor-beta

TNF-α	tumour necrosis factor-alpha (cachexin)
TNF-β	tumour necrosis factor-beta (lymphotoxin)
UTR	untranslated region
V_A	additive variance
V_D	dominance variance
VDR	Vitamin D Receptor
$1,25(OH)_2D_3$	1a,25-dihydroxycholecalciferol
VDRE	Vitamin D response element
VOS	velocity of sound

Chapter 1: Introduction

1.1 Osteoporosis: Definition and Epidemiology

Osteoporosis is a systemic metabolic bone disease characterised by decreased bone mass and microarchitectural changes, resulting in increased fragility of bone and a propensity to fracture (Consensus development conference V 1994). Osteoporosis may result in fracture of any bone, however the most common sites are bones with high trabecular bone content – the vertebrae, hip and distal radius. Osteoporotic fracture rates increase exponentially with age in both men and women (Melton 1988). However, there is approximately a two-fold higher incidence of fracture in women compared with men for all age-related fracture rates, mainly due to postmenopausal accelerated bone loss.

Osteoporosis is the most prevalent metabolic bone disease in the developed world and is becoming increasingly prevalent in the developing world. Hip fractures are the most serious clinical outcome of osteoporosis, because they account for most of the mortality, morbidity and costs of the disease. There are substantial race and sex differences to hip fracture rates. In American Caucasians aged 50 years, the remaining lifetime risk of hip fracture is 11-18% in women and 6% in men (Melton et al. 1992), with approximately a 2:1 female to male fracture ratio. A similar female-to-male fracture ratio has been reported in Australian Caucasians (Jones et al. 1994). Hip fracture rates are lowest in black populations, with about one third of the risk of hip fracture for Caucasians, with rates in Asian populations about half the Caucasian rate (Genant et al. 1999). The female to male ratio of fracture in these populations is lower than in Caucasian populations. Hip fracture rates are expected to quadruple worldwide from an estimated 1.66 million hip

fractures in 1990 to 6.26 million by 2050, with approximately 75% expected to occur in the developing world of Asia and Latin America (Cooper et al. 1992). Thus an increasing proportion of fractures will occur in men, in whom – at least in Caucasian populations – mortality after a hip fracture is higher than for women (Center et al. 1999; Forsen et al. 1999; Jacobsen et al. 1992).

Vertebral fracture incidence is more difficult to estimate, as many vertebral fractures do not come to clinical attention. Using radiographic evidence of vertebral deformity to indicate vertebral fracture, prevalence rates have been estimated overall at 12% in European males and females aged over 50 years (O'Neill et al. 1996). Prevalence also increased with age in both sexes, though more steeply in females than in males, and fracture rates were higher in more Northern countries. Slightly higher figures were reported in a North American study, with marked increase in vertebral fracture prevalence with age (Cauley et al. 2000). An Australian study found that at least 17% of males and 12% of females aged 60 years or older have vertebral deformity, and the overall residual lifetime risk of any fracture (the bulk of which are fractures of the hip, vertebrae, humerus and distal radius) for a person aged 60 years with average life expectancy is 29% in males and 56% in females (Jones et al. 1994).

Mortality is increased after osteoporotic fracture in both men and women, with greater mortality in males following all types of osteoporotic fracture compared with females. A large Norwegian study showed mortality rates for hip fracture of 31% in males and 17% in females (Forsen et al. 1999). Although most deaths occurred in the first year after fracture, the excess mortality in fracture patients persisted for at least 5 years. The

relative risk of dying for patients with hip fracture compared with controls was 4.2 for men and 3.3 for women. Age-adjusted mortality ratios following hip fracture in an Australian study were somewhat lower at 3.17 for males and 2.18 for females (Center et al. 1999). Of note, excess mortality from hip fracture was evident in all age groups, but was highest in younger patients (Center et al. 1999).

Increased mortality also occurs in patients with clinically diagnosed vertebral fractures. The only study to assess mortality after clinically evident vertebral fracture in both men and women demonstrated age-adjusted mortality ratios of 2.38 for males and 1.66 for women (Center et al. 1999). The result for women concords with a previously published study of mortality after vertebral fracture in women (Cooper et al. 1993). Both these studies suggested that increased mortality after vertebral fracture might be due to co-morbid conditions increasing the risk of both death and osteoporotic fracture, rather than necessarily arising from vertebral fracture *per se*. Patients with radiographic evidence of vertebral deformity also have reduced survival, with excess mortality rate ratios of 1.9 in women and 1.3 in men (Ismail et al. 1998) but after adjustment for adverse health and lifestyle factors, the excess mortality rates were not significant in either sex, supporting the concept that excess mortality observed in patients with vertebral fractures may be due to other factors.

Morbidity is also highest from hip fractures. Acute complications include pressure sores, urinary tract infections, pneumonia, anaemia, and operative and anaesthetic complications, in addition to the pain of the fracture. Chronic complications include loss of mobility (of the 80% of patients ambulant before hip fracture, 50% are unable to walk

independently afterwards), loss of independence and a high risk of institutionalisation. Morbidity from other osteoporotic fractures is also not inconsiderable, with vertebral fracture resulting in back pain, kyphosis, height loss, and loss of quality of life (reviewed in (Cooper 1993)).

1.2 Clinical Picture

Osteoporosis is a clinically heterogenous condition and can result from more than one pathological process. It can be divided into primary (or idiopathic) osteoporosis, and secondary osteoporosis, resulting from a number of causes (see Table 1.2). Historically, primary osteoporosis was previously further subdivided into type 1 osteoporosis, referring to postmenopausal osteoporosis in women, and type 2 osteoporosis, bone loss due to ageing, occurring in both men and women. However, this subdivision is somewhat artificial, as there is no histological difference between the two types. Additionally, this model implies that osteoporosis is solely a disease of loss of bone whereas bone mass at any one point in time is the result of peak bone mass achieved and subsequent bony loss.

Peak bone mass is achieved in both sexes after a period of rapid pre-pubertal bone growth with a post-pubertal period of consolidation lasting approximately a decade. The greater peak bone mass observed in men is mainly due to greater bone size (Henry et al. 2000; Orwoll et al. 1995) and true volumetric bone mineral density (BMD) may not differ between the sexes or may even be higher in women (Seeman 1995). BMD is thought to

be maximal between the ages of 20 and 30 years, after which bony loss due to ageing becomes noticeable. Peak bone mass predominates in determining overall bone mass until late in life when bone loss becomes increasingly important. At age 65 years, peak bone mass and bone loss are thought to contribute equally to the overall variance of BMD, but by age 80 years bone loss accounts for approximately 80% of the variance in BMD (Cooper 1997). It is not clear whether the mechanism by which a person has low BMD – either failure to achieve good peak bone mass, or subsequent excessive bone loss – has any bearing upon fracture risk. However some studies have shown that markers of bone resorption predict hip fractures independently of BMD (Garnero et al. 1996; Melton et al. 1997), suggesting that increased bone turnover *per se* is a risk factor for fracture, whilst an isolated longitudinal study of forearm BMD in women suggested that rapid bone loss contributes as much to risk of fracture as does low BMD (Riis et al. 1996).

Table 1.2: Secondary Causes of Osteoporosis

Secondary Causes of Osteoporosis	**Examples**
Endocrine disorders	Hypogonadism (primary or secondary) Hyperthyroidism Hypercalciuria Cushing's syndrome Hyperparathyroidism
Malignancy and infiltrative conditions	Multiple myeloma, leukaemias and lymphomas Waldenstrom's macroglobulinaemia Mastocytosis
Gastrointestinal disorders	Malabsorption of any cause, including coeliac disease Gastrectomy (especially males) Chronic liver disease
Drugs	Glucocorticoid therapy Anticonvulsant therapy Heparin Excessive thyroxine replacement Protease inhibitors
Renal disorders	Renal tubular acidosis Chronic renal failure
Cigarette smoking	
Inflammatory conditions	Rheumatoid arthritis Ankylosing spondylitis
Dietary	Vitamin D deficiency Calcium deficiency Alcoholism (N.b. Modest alcohol intake is not associated with low BMD or fracture)

1.2.1 Bone Mineral Density

The major clinical outcome of osteoporosis is fracture. Fracture risk clearly depends on bone fragility but also upon other factors, principally exposure to trauma, which are difficult to measure and control for in analysis. Further, fracture incidence rates are quite low, making collection of sufficient clinical resources for research purposes both difficult and expensive. Therefore whilst the study of fracture rates has the major advantage of direct biological relevance, measuring the amount of bone tissue is much easier and more precise, and thus low BMD is frequently used as a surrogate or synonym for osteoporosis, especially for clinical and genetic research.

The close association between BMD measured by densitometry at hip or spine and subsequent fracture risk of both sites has been shown in longitudinal and cross-sectional studies in both men and women. Prospective studies have shown that the risk of fracture in general increases with decreasing BMD, regardless of measurement site, with overall fracture risk approximately doubling for each standard deviation (SD) decrease in bone mineral density (Genant et al. 1999; Wasnich 1993). However, the relationship between site-specific BMD measurement and subsequent fracture of that site is even greater. Cummings and colleagues found the age-adjusted risk of hip fracture per SD decrease in femoral neck (FN) bone density was 2.6 in women, with BMD measurement at FN a better predictor than BMD at lumbar spine (LS) or radius, although low BMD at LS and radius also resulted in an increased risk of hip fracture, with risk of 1.6 per SD decrease in BMD (Cummings et al. 1993). A large prospective study in the Netherlands recently showed that the relative risk of hip fracture per SD decrease in femoral neck BMD was

2.5 for women, and 3 for men (De Laet et al. 1998). A recent Meta-analysis of 7 prospective studies measuring BMD at various sites reported the highest relative risk of subsequent hip fracture per SD decrease in BMD at femoral neck and trochanter (RR of 2.29 and 2.22 respectively), although the analysis also found that BMD at any site was predictive of subsequent hip fracture (Woodhouse et al. 2000). These results concorded with a previous Meta-analysis of the overall and site-specific predictive value of BMD (Marshall et al. 1996). Conversely, patients with fracture have been found to have generalised osteopaenia with BMD lowest at the site of fracture (Aloia et al. 1992; Chevalley et al. 1991; Firooznia et al. 1986). A study of male patients with either vertebral or femoral neck fracture found site specificity of diminution of both bone size and BMD (Seeman et al. 2001). In addition to clinical data, mechanical testing of failure load for cadaveric bone samples of femur, vertebra and calcaneus is highly correlated with site-specific BMD measured by dual energy X-ray absorptiometry (DXA), with somewhat weaker although still positive correlations with BMD measurements at other sites (Cheng et al. 1998).

The correlation between BMD measured at different sites is closer in the younger healthy population than in patients with significant bone loss. As rates of bone loss differ at different sites, it is not surprising that the correlation of BMD between sites declines with age (Kanis et al. 1994), and therefore site-specificity of BMD measurement and fracture prediction may increase. Additionally, BMD measurements may become less accurate with age at some sites such as the lumbar spine, where aortic calcification, spinal osteoarthritis and the presence of osteophytes bias projectional BMD measurements (see below). Indeed, age-related bone loss, which proceeds at all sites after about the age of

30 years, may not be measurable at the lumbar spine in the elderly because of the increase in the presence of confounders (Brown et al. 2001; Burger et al. 1994) in both men and women. Lateral DXA scanning can provide a BMD reading free of the effect of such confounders. Overall, bone loss with ageing is higher in women than in men (Hannan et al. 2000).

The World Health Organisation has defined osteoporosis in white women as BMD 2.5 SD below the mean for a young healthy population (a t-score of –2.5 or below). Osteopaenia is defined as BMD with t-score between –1 and –2.5 (Kanis et al. 1994). Of those women who fracture, 95% will have BMD below the –2.5 SD threshold (Maricic et al. 2000); and the prevalence of osteoporosis defined by this threshold approximates the prevalence of osteoporotic fracture (Kanis et al. 1994). Women with osteopaenia are also at increased risk of fracture, and intervention to improve BMD will lessen their fracture risk. Defining osteoporosis by BMD values allows identification of a patient cohort with low BMD, whether the cause is failure to achieve good peak bone mass or rapid bony loss. It does not help with planning preventative measures.

The appropriate BMD values to define osteoporosis and osteopaenia in males or in non-Caucasian populations are not known. Melton and colleagues, having formulated a male normative data base locally, found that using a threshold of a t-score of –2.5 at either hip or spine resulted in prevalence rates of BMD-defined osteoporosis of 19% of males over 50, an overestimate of the actual osteoporotic fracture rate that they observed for males of 13% (Melton et al. 1998). Other studies have found a higher prevalence of osteoporotic fracture in men, some with estimates as high as 25% of males aged over 60 years

(Nguyen et al. 1996). However, using the normative database provided by the manufacturer for each type of densitometry machine (excluding CT scanning) Faulkner and Orwoll found 4-9% of males with a t-score less than –2.5, a substantial under-representation of the group who will fracture (Faulkner et al. 2000). Using a t-score of –2 resulted in a prevalence of osteoporosis as defined by densitometry measurements that more closely correlated with fracture prevalence, and thus provided a better identification of the group who would benefit from intervention. Use of a t-score less than –2.5 using the normative database established by the US National Health and Nutrition Examination Survey (NHANES III) also underestimated the frequency of male osteoporotic fracture (Looker et al. 1997). The use of a male-derived cut-off value (lowest quartile of BMD) correlated better with vertebral fracture prevalence than the use of female-derived cut-offs (Cauley et al. 2000). Thus further epidemiological work is needed to determine the appropriate use of BMD to define osteoporosis in men.

1.2.2 Measurement of BMD by Dual Energy X-ray Absorptiometry

BMD measurement can be performed by a number of techniques. True volumetric bone density can be measured by computerised tomography (CT) scanning, which also allows differentiation of cortical and trabecular bone. CT scanning involves substantial radiation exposure and is thus unacceptable as a means of monitoring BMD as many repeat scans may be needed. Absorption of either X-rays from a cathode ray tube or photons from degenerating I^{131} is the common method of assessing BMD. BMD can be assessed at the wrist using single energy photon or X-ray absorption, or at the lumbar spine and femur using dual energy photon or X-ray absorption. These sites, the most common sites of

osteoporotic fracture, are rich in trabecular bone. Although overall trabecular bone accounts for only 15% of the skeleton, the lumbar spine consists of over 75% trabecular bone, the intertrochanteric area of the femur 50%, the femoral neck 25%, and the distal radius 25% (Mundy 1999).

DXA is widely used in the assessment of osteoporosis because of its rapidity, low dose of ionising radiation and high precision (coefficient of variation of 0.5-1.5%) (Genant et al. 1996). DXA scanning of the vertebral bodies of the lumbar spine and the femur involves a projectional technique in which total calcium content (calculated by the absorption of X-rays) is divided by the area of the bone. This results in an areal bone mineral density rather than a true volumetric measurement. Such a result is biased by the size of bones in that a larger bone will appear to have higher BMD due to the greater depth of bone in the area scanned. In general this is not considered in clinical practice. If bone size itself is an important predictor of bone strength (and bone size is predictive of fracture independently of BMD (Gilsanz et al. 1993)) then adjusting BMD for measures of bone size will reduce the information about fracture risk, whereas areal BMD captures both facets of bone size and bone density (Compston 1995). However, both areal bone density and estimated volumetric bone density of the hip have been shown to have very similar predictive values for hip fracture *in vivo* (Cummings et al. 1994) and *ex vivo* (Tabensky et al. 1996). Further, measurement of areal BMD will underestimate the true bone density loss with ageing, due to periosteal appositional growth continuing throughout life and increasing bone dimensions (Kanis et al. 1994).

Carter and colleagues proposed several methods of calculating a true volumetric bone density (measured in g/cm^3), which they called bone mineral apparent density (BMAD) (Carter et al. 1992). In order to calculate BMAD, approximations for bone thickness and hence bone volume were considered. Candidates that would allow adjustment of BMD to an anthropomorphic measure that would reflect bone thickness included height; the square root of observed bone area (which the authors demonstrated was also proportional to height); and observed bone width (although this information is not usually given in bone densitometry reports; however, it can be formally measured by the investigator from the printed output picture of the bone in question). Using these approximations, Carter and colleagues demonstrated that BMAD was independent of height and weight. As mentioned above, the importance of BMAD or other measures accounting for bone size is not so much in the clinical context (where an individual may have repeat BMD to follow the development and/or treatment of osteoporosis) as in the research setting, where comparison of BMD of individuals of different sizes may be inherently biased. This is discussed further in Chapters 3 and 4. Some authors have argued that the observed differences in BMD between men and women are due to sexual dimorphism in skeletal size: once BMD is corrected for bone size (using BMAD or other measure), that men and women have skeletons of equal volumetric bone density (Seeman, 2001).

Lumbar spine DXA measurements may be affected by age-related degenerative disorders such as aortic calcification, lumbar spondylosis, osteoarthritis, or previous lumbar vertebral fracture, all of which may artificially elevate bone density measures without an associated increase in bone strength. These various measurement problems all add further variability to BMD results, reducing the correlation of fracture risk and bone density

measurement and the power of genetic studies using DXA measures to identify osteoporosis genes.

BMD can also be measured by quantitative ultrasound (QUS), which is influenced by both bone mineral content and bone microarchitecture. QUS measurements may predict fracture risk independently from DXA at both LS (Bauer et al. 1995; Cepollaro et al. 1997) and hip (Bauer et al. 1997), which may relate to effects of bone microarchitecture upon QUS (Wu et al. 1998). Additionally some QUS machines are capable of specifically examining cortical bone rather than the mix of cortical and cancellous bone measured by DXA. Thus genetic studies using QUS may detect genetic effects upon fracture risk not detectable by DXA scanning (Patel et al. 2000).

1.3 Epidemiological Risk Factors for Osteoporosis

Large prospective studies have identified a number of risk fractures for hip fracture for women. Cummings and colleagues have reported a large prospective study of risk factors for hip fracture in white women (Cummings et al. 1995). Maternal history of hip fracture was second only to use of anticonvulsant medications as the strongest risk factor for hip fracture, with a relative risk of 2.0. If the woman's mother fractured before age 80, the relative risk increased to 2.7. Adjustment for BMD and history of previous fracture slightly reduced the relative risk to 1.8. Low BMD at the calcaneus and previous fracture after age 50 independently increased the relative risk of hip fracture (1.6 and 1.2 respectively). Weight was also an important determinant of fracture risk. Women who

weighed less than they had at 25 had a relative risk of hip fracture of 2.2, whereas the more weight a woman had gained since age 25 the lower her risk of hip fracture. A number of factors associated with fitness and neuromuscular strength were significant in determining hip fracture. Women who spent less than 4 hours a day on their feet had twice the risk of fracture compared with women who spent more than 4 hours on their feet, and women who walked for exercise had a 30% lower risk of hip fracture compared with non-exercisers. An inability to rise from a chair without using one's arms (relative risk 2.1) and visual difficulties such as poor depth perception (1.5) or low contrast sensitivity (1.2) – all features associated with increased risk of falling – were (not surprisingly) associated with increased fracture risk. Other significant factors included raised resting pulse rate (1.8), a history of hyperthyroidism (1.8), use of anticonvulsant medication (2.8) or long-acting benzodiazepines (1.6), and women who rated their own health as poor (1.7). Current smokers had twice the risk of hip fracture compared with non-smokers or ex-smokers; however the smokers also had lower weight, poorer self-health rating, were less likely to exercise or spend time on their feet and had relative tachycardia, and after multivariate adjustment smoking was no longer a significant independent risk factor. Modest alcohol intake (7 drinks a week or less) was associated with a lower risk of fracture, although overall the protective effect of alcohol ingestion (relative risk of 0.7) was lost once adjusted for better self-reported health and the ability to stand up from a chair (Cummings et al. 1995). Hypogonadism induced by menopause was not included in this study, but is a key factor in the development of osteoporosis (Black et al. 2000). A history of previous osteoporotic fracture was also not examined in this study, but is a major risk factor for hip fracture, with prevalence of 34% in elderly

women with hip fracture in northern Britain (Stewart et al. 2000). Estimated risk of hip fracture recurrence after previous hip fracture was 29% in a Minnosotan population of both men and women, 1.6 times greater than expected (Melton et al. 1982). Many of the risk factors for hip fracture also increase the risk of osteoporotic fracture at other sites.

Many of the risk factors for fracture in males are similar to those in females, and include low BMD, risk of falling, physical inactivity, hypogonadism, use of glucocorticoid and anticonvulsant medication, alcoholism, hyperthyroidism, cigarette smoking, gastrectomy, malabsorption, and hypercalciuria (reviewed in (Eastell et al. 1998; Orwoll et al. 1995)) with hypogonadism and glucocorticoid usage common secondary causes of osteoporosis and fracture in men. Genetic factors contribute to fractures in males as in females (Krall et al. 1993).

Body mass index is a strong predictor of BMD at all sites of measurement and in both sexes (Burger et al. 1994; Eastell et al. 1998; Felson et al. 1993). Low body mass index is negatively correlated with peak bone mass, and low body mass index and weight loss are strongly associated with increased fracture risk (Black et al. 2000).

The concept that environmental influence at critical periods of early development might have lasting and longterm effects upon health is referred to as programming, and was initially proposed by Barker (1995) with respect to ischaemic heart disease. There is some evidence that intrauterine and early postnatal growth may be associated with BMD in later life. Weight in early infancy (age 1 year) has been found to correlate with bone mineral content (though not BMD) in late adult life (Cooper et al. 1997). More recently, birth weight was found to correlate with bone size, bone mineral content and BMD at LS,

FN and whole body, although when current weight was added in as a covariate, the association between birth weight and bone mineral content became non-significant and the results for bone size and BMD were not reported (Gale et al. 2001).

1.4 Genetic Epidemiology of Osteoporosis:

1.4.1 Quantitative Traits

The normal distribution exhibited by many biological traits, including BMD, may result from the inheritance of many individual loci (Fisher 1918). The term Quantitative Trait Loci (QTL) is now used for the genes determining such normally distributed traits. Quantitative traits may result from the action of allelic variation of multiple genes or from the action of a single or few gene(s) with strong environmental influence upon the final phenotype.

The model proposed by Fisher specified that any continuous phenotype (P) could be considered as a function of both the effects of genes (G) and the environment (E).

$$P = E + G$$

This concept can be extended to analysis of variance, such that total phenotypic variance (V_P) is due to both environmental (V_E) and genetic (V_G) variances.

$$V_P = V_E + V_G$$

Additionally, the total genetic variance of a trait (V_G) can be split into additive (V_A) and dominance (V_D) effects. Additive effects are due to the action of individual alleles; dominance effects are due to the interaction between alleles. The assessment of additive and dominance variance is affected by epistasis, or non-allelic interaction between genes, and by gene-environment interaction and/or correlation. Imprinting effects upon alleles may also exist (see Chapter 6). Variance of a trait may also be split into factors shared by members of families and/or the general population (both shared genetic and shared environmental influences), and factors pertaining only to the individual (non-shared environment and unique individual characteristics). This is discussed further below (see Section 1.5.1).

Heritability refers to the proportion of the total variance of a trait that is under genetic control, or in other words the amount of variance that is heritable. Heritability can be split into broad sense heritability, the overall genetic contribution to the variance of a trait, and narrow sense heritability, the additive component of the total heritability (Kearsey et al. 1996).

$$\text{Broad sense heritability} = (V_A + V_D) / (V_A + V_D + V_E)$$

$$\text{Narrow sense heritability} = V_A / (V_A + V_D + V_E).$$

1.4.2 Heritability Studies of BMD and Osteoporosis

Heritability studies of BMD have been performed in both twin and intergenerational studies, showing heritability of BMD to be high.

Heritability studies in twins rely on a number of assumptions. Monozygotic (MZ) twins are assumed to share 100% of their genes whilst dizygotic (DZ) twins share on average only 50%. Twin models assume that the within-pair environmental variance is the same in MZ and DZ twins (i.e. that MZ twins are no more likely to share environmental characteristics than are DZ twins). The models also assume that the total MZ and DZ variances are equal, that there is no gene-gene interaction (neither dominance nor epistasis), and that there is neither gene-environment interaction nor correlation. With these assumptions in mind, twin studies partition the total variance into between-pairs and within-pairs variance, and then compare the within-pair variance in MZ twins with that in DZ twins. Any difference seen is assumed to be due to the greater genetic sharing of MZ twins (Christian et al. 1974; Falconer 1964). However, the greater sharing of environmental factors by MZ twins (and the likelihood of gene-gene interaction) mean that estimates of heritability in twin studies may be inflated (Slemenda et al. 1991). To balance this, DZ twins may be genetically more similar than expected (greater than 50% IBD used in twin heritability studies) (Brown et al. 1997; Jawaheer et al. 1996).

Mixed sex twin studies have found heritability of BMD at the femoral neck and lumbar spine to be similar, between 57-92% (Nguyen et al. 1998; Pocock et al. 1987; Smith et al. 1973), whilst in general forearm BMD has been demonstrated to have lower heritability (Arden et al. 1996; Flicker et al. 1995; Pocock et al. 1987; Slemenda et al. 1991; Smith et al. 1973). Whilst several studies performed in female twins have shown BMD to be highly heritable (Arden et al. 1996; Flicker et al. 1995; Howard et al. 1998; Slemenda et al. 1991), there is a paucity of data regarding male twins alone (Smith et al. 1973), making comparisons between the genders difficult. Some authors have suggested that

heritability may be greater in premenopausal than postmenopausal women (Arden et al. 1996; Slemenda et al. 1991), although there has been no direct comparison of these two groups. However, the within-twin variance of female MZ twins increases with age, suggesting an accumulation of environmental influences with ageing in women, (Slemenda et al. 1991) consistent with but not limited to the effects of menopause. Additionally, the variance of both MZ and DZ twins increases with ageing in men (Slemenda et al. 1992), consistent with increasing environmental effects upon BMD in men also.

Familial intergenerational studies generally have estimated heritability of BMD to be somewhat lower than reported in twin studies, though still substantial (heritability estimates of 0.46-0.84 for total BMD and for BMD measured at individual sites of the femur, lumbar spine and forearm) (Deng et al. 1999; Gueguen et al. 1995; Krall et al. 1993; Sowers et al. 1992). However, environmental influences upon bone may differ between siblings and across generations considerably more than within either MZ or DZ twin pairs. This may be particularly the case when comparing BMD across generations with different maturity and endocrine effects upon the skeleton. Thus it is not surprising that heritability estimates from family studies are somewhat lower than in twin studies.

The strong correlation of BMD between parents and children indicates that peak bone mass is a major determinant of BMD in later life (Jones et al. 2000; Jouanny et al. 1995). Children and other young relatives of patients with a history of low trauma fracture have low BMD (Cohen-Salal et al. 1998; Evans et al. 1988; Seeman et al. 1989; Seeman et al. 1994), indicating that bone fragility in older age is at least partially determined by low

peak bone mass. Heritability of peak bone mass may be the greatest component of overall heritability of BMD. This is further supported by the demonstration that the maximum value of heritability is achieved at 26.4 years of age (Gueguen et al. 1995), and that the correlation in BMD is stronger between mothers and their younger premenopausal daughters than their older post-menopausal daughters (Danielson et al. 1999).

BMD in both sons and daughters correlates most closely with the mid-parental value, indicating that both parents make a genetic contribution to BMD, consistent with a polygenic or co-dominant monogenic mechanism of disease (Krall et al. 1993). A segregation study by Gueguen and colleagues (Gueguen et al. 1995) also supported a genetic model of polygenic inheritance of BMD. Further, the demonstration of correlation of BMD between different gender parents and children supports the existence of at least some common genetic determinants of BMD in males and females (Jones et al. 2000; Jouanny et al. 1995; Krall et al. 1993). In addition, however, heritability estimates of LS, FN and total BMD using mother-daughter pairs were found to be significantly greater than those derived from mother-son pairs, supporting the concept of some gender-specific genetic effects upon BMD (Jones et al. 2000). A marginally stronger correlation was demonstrated between the BMD of children and that of their mother rather than father (Jouanny et al. 1995; Krall et al. 1993), probably due to greater similarity of lifestyle factors (Krall et al. 1993).

Heritability of BMD may be site-specific (Deng et al. 2000; Nguyen et al. 1998). Daughters of mothers with a history of osteoporotic lumbar spine fracture have lower

BMD at the spine than at other sites (Seeman et al. 1989). Daughters of mothers with femoral neck fractures only had low BMD at the femur (Seeman et al. 1994). Site-specificity of inheritance of fracture risk also supports this concept (Fox et al. 1998). Alternatively, gene-environment interaction may be different at different anatomical sites (Deng et al. 2000).

Tabensky and colleagues have recently examined the heritability of both calculated volumetric BMD (vBMD) and bone volume of both LS and FN in women with vertebral or femoral fractures and their daughters, compared with normal women (Tabensky et al. 2001). Whilst women with vertebral fracture had both reduced vBMD and reduced vertebral size, their daughters had reduced vBMD only and had normal vertebral size. The authors conclude that vBMD at LS is genetically determined and due to effects upon peak bone mass, and that bone size at LS is determined more by age-related bone loss than peak bone mass. This explanation can be extended to argue that vBMD at LS is under greater genetic control than is vertebral volume. Women with femoral fractures had reduced vBMD but increased bone volume. Their daughters had normal vBMD but also had increased bone volume. The authors conclude that low BMD at FN was more due to age-related bone loss that to reduced peak bone mass, and that increased bone volume at this site was due to genetic factors. It is interesting to contrast these results at FN with previous work by one of the authors, demonstrating reduced BMD at FN in daughters of women with hip fracture (Seeman et al. 1994), and to evidence that small bones are more susceptible to fracture (Gilsenz et al 1993).

Twin studies of heritability of quantitative ultrasound (QUS) have demonstrated that QUS is also a highly heritable trait, with estimates of 0.74 for broadband ultrasound attenuation (BUA), 0.55 for velocity of sound, and 0.82 for speed of sound (Howard et al. 1998). Adjustment for DXA-measured BMD had only a minor reduction on heritability of QUS, suggesting that there may be different genetic effects upon BMD measured by DXA and QUS (Arden et al. 1996). In contrast to that observed with BMD measured by DXA, BUA was more strongly correlated between mothers and their postmenopausal rather than premenopausal daughters (Danielson et al. 1999). Thus the genetic contribution to qualitative changes in bone induced by menopause (as measured by QUS) may differ from the genetic determination of bone mass *per se*.

In addition to the heritability of peak bone mass, some twin and family studies have also suggested heritability of bone turnover and loss. Significant heritability of markers of bone turnover (both synthesis and degradation) has been demonstrated in twins and families (Garnero et al. 1996; Hansen et al. 1992; Harris et al. 1998; Hunter et al. 2001; Kelly et al. 1991; Tokita et al. 1994). However, there have been few studies of the heritability of bone loss conducted in a prospective longitudinal fashion. Significant heritability of bone loss over three years was demonstrated at LS and Ward's triangle but not at other hip sites in one twin study (Kelly et al. 1993). No heritable effect on radial bone loss was demonstrated in a study of elderly male twins (Christian et al. 1989). In general, estimates of heritability of bone turnover and of bone loss have been lower than estimates of heritability of peak bone mass.

Despite the strong evidence of heritability of BMD, a large Finnish study following over 15000 elderly twins of both genders was not able to detect heritability of fracture *per se* (Kannus et al. 1999). The numbers of concordant pairs of twins with fractures were very small (for example, only 8 male MZ twin pairs and 4 male DZ twin pairs were concordant for fracture). A higher concordance of fracture in MZ twins compared with DZ twins was found in both male and female twin pairs but these results were not significant (Kannus et al. 1999). Other authors have suggested that the data presented did demonstrate heritability of fracture risk in men, with heritability estimates of 35% (MacGregor et al. 2000).

In summary, BMD is a heritable quantitative trait. Peak bone mass may be the major component under genetic control, although there is some evidence of heritability of bone turnover and bone loss. Heritability of BMD and fracture may differ at different skeletal sites, either due to effects of different genes upon different sites or to differing gene-environment interaction. There may be both common and gender-specific effects upon BMD, which again may be site-specific. Thus osteoporosis is a complex disease with polygenic inheritance influenced by gene-environment interactions, and determination of disease-causing genes in this illness will be difficult.

1.5 Statistics for Human Genetics

1.5.1 Linkage

Genetic linkage refers to the co-segregation of a disease-associated allele and a marker allele within families. A disease-associated allele at a given locus lying close to a marker will be inherited with that marker in a family, unless a recombination event occurs. The likelihood that the marker and mutation will stay on the same haplotype (chromosomal strand) depends on the distance between the two and the number of meioses studied. The chance of recombination between two loci in any given meiosis is the recombination fraction (θ). A recombination rate of 1% (θ=0.01) corresponds to a genetic distance of one centiMorgan (1cM), which is approximately equivalent in most regions of the genome to a physical distance of one million base pairs (1 Mb). The relationship between map distance and the recombination rate (θ) is described by mapping functions such as Haldane and Kosambi functions. The map length of a chromosome is the average number of crossovers in the interval of a single chromatid. The Haldane function assumes that recombination can occur at equal probability anywhere between two loci, whilst the Kosambi function takes into account genetic interference, i.e. the suppression of recombination in the regions of DNA close to a recent recombination event. The Kosambi map function can be expressed as:

$$\theta = 1/2\ (e^{4m} - 1)/(e^{4m} + 1)$$

where θ = recombination fraction and m = map distance (Kosambi 1944).

Relatively few meioses separate family members within an individual family compared with the number of meioses dividing distantly related individuals such as members of the general population. Therefore when using families in linkage studies, the distance between the marker and mutation can be quite large, yet linkage may still be observed, as few recombination events will have occurred within each individual family. Linkage is always the result of association (or linkage disequilibrium) between two loci (a marker allele and the disease-causing allele), however that association may be only intrafamilial and not evident at a population level (Hodge et al. 1994).

Linkage analysis methods can be broadly divided into parametric (or model-based, or LOD-score) methods; and non-parametric (or model-free) methods.

Parametric linkage analysis methods are based on the likelihood (odds) ratio of L_{HA}/L_{H0} where L_{H0} (the null hypothesis) is the likelihood of no linkage (i.e. $\theta = 0.5$) and L_{HA} is the likelihood that there is linkage (i.e. $\theta < 0.5$). For a given set of genotypic data, the likelihood of producing the data can be calculated according to certain assumptions or parameters (including mode of inheritance of the trait, allele frequencies of the loci, penetrance of the trait, no inbreeding of the population, no phenocopies in the population, and no pleiotropy). This likelihood ratio can be converted to a 'LOD' score by specifying the prior probability of linkage (Elston 1997) (conventionally estimated at 1/50), and a maximum LOD score (MLS) can be calculated by testing across different values of θ. If a LOD score is maximised over a single unknown parameter (i.e. θ), a LOD score of 3 corresponding to a p-value of 10^{-3} has been conventionally regarded as 'significant'. A LOD score below –2 is thought sufficient to exclude linkage. Lander

and Kruglyak have recently shown that for parametric analysis, an exact genome-wide significance threshold of $p = 0.05$ is achieved at a pointwise p-value of 4.9×10^{-5} or LOD of 3.3 (Lander et al. 1995). Additionally, model mis-specification may greatly diminish the power of model-based linkage studies (Clerget-Darpoux et al. 1986). However, where the parameters are accurately known (or where there are good estimates for missing parameters), model-based linkage analysis is the most powerful linkage analysis technique (Elston 1998; Jarvik 1998; Nyholt 2000).

For complex diseases such as osteoporosis, though, many of the parameters needed for LOD-score based approaches are not known. Instead, model-free or non-parametric approaches can be used, which do not require the mode of inheritance of a trait to be specified but rely upon known modes of marker inheritance. Broadly speaking, the major types of model-free (or non-parametric) analysis can be split into allele sharing methods (e.g. Affected Sibling Pair methods (ASP), Affected Pedigree methods, the Haseman-Elston statistic) and variance components methods.

The most commonly used allele sharing methods is the affected sib-pair method (Penrose 1935). By chance, two affected siblings share 0, 1 or 2 marker alleles identical by descent (IBD) in the ratio 0.25: 0.5: 0.25. If the marker locus under consideration is linked to the disease-causing locus, then the allele sharing will be skewed away from sharing 0 alleles IBD towards sharing 1 or 2. In other words, two siblings identical in phenotype would be genotypically more similar at that marker than would be predicted by chance alone. IBD sharing means that alleles shared by relatives are not just identical by state but can be identified as coming from the same familial ancestral chromosome.

For siblings this usually means that parental genotypes must be known to establish IBD sharing. It may be possible to infer missing parental genotypes and parental phase from other members of the family, e.g. using the genotypes of other siblings. If IBD sharing cannot be established unequivocally then likelihood methods can be used to estimate IBD sharing (Risch 1990).

The appropriate threshold of significance for ASP methods is higher than for LOD score analysis. LOD score analysis examines inheritance from two parents to one child, whilst ASP methods involve inheritance to two children. Lander and Kruglyak have argued therefore that for ASP at a genome-wide level, evidence of suggestive linkage requires a point p-value of 7.4×10^{-4} (a LOD of 2.2) and significant linkage 2.2×10^{-5} (LOD=3.6) (Lander et al. 1995).

In addition to ASP methods, another popular allele sharing method is the affected pedigree method, using vector-descent pathways to estimate IBD sharing between relatives (Weeks et al. 1988). Alternatively, LOD-score or parametric analysis is sometimes used even when the mode of inheritance is not known, as it has been demonstrated that as long as this parameter is approximately correct the LOD-score methods are more powerful than nonparametric models (Nyholt 2000).

Allele-sharing methods can be extended to quantitative trait analysis. Instead of using affected siblings in a qualitative fashion, the squared difference between the trait values for the two siblings is regressed against allele sharing IBD at a marker (Haseman et al. 1972) (see Diagram 1.5.1). If the marker locus is linked with a quantitative trait locus, then siblings with greater allele sharing at the marker will be concordant for the trait, and

siblings with lesser allele sharing will be phenotypically discordant. Thus the regression line will have a negative gradient. Conversely, if the marker locus is not linked with the quantitative trait locus then the gradient of the regression line will be nought, as the phenotypic concordance between the siblings will be independent of allele sharing at the marker locus.

In the presence of linkage, the steepness of the negative gradient of the regression line will be greatest (and hence of greatest significance) if the siblings are chosen for extreme concordance or discordance (Risch et al. 1995). A qualitative or binary disease may be analysed by the Haseman-Elston algorithm by giving the value 0 and 1 to unaffected and affected persons respectively. In this case, the test becomes a comparison between the mean proportion of allele sharing in discordant and concordant siblings. If only extremely discordant or extremely concordant siblings are available, the mean allele sharing IBD can be compared with that expected by chance alone (50%); in the presence of linkage the observed sharing would be lower for extremely discordant sibs, and higher for concordant sibs (Elston 1998). (The use of siblings and probands with extreme trait values is discussed further in Chapters 3 and 4).

Diagram 1.5.1: Haseman-Elston statistics

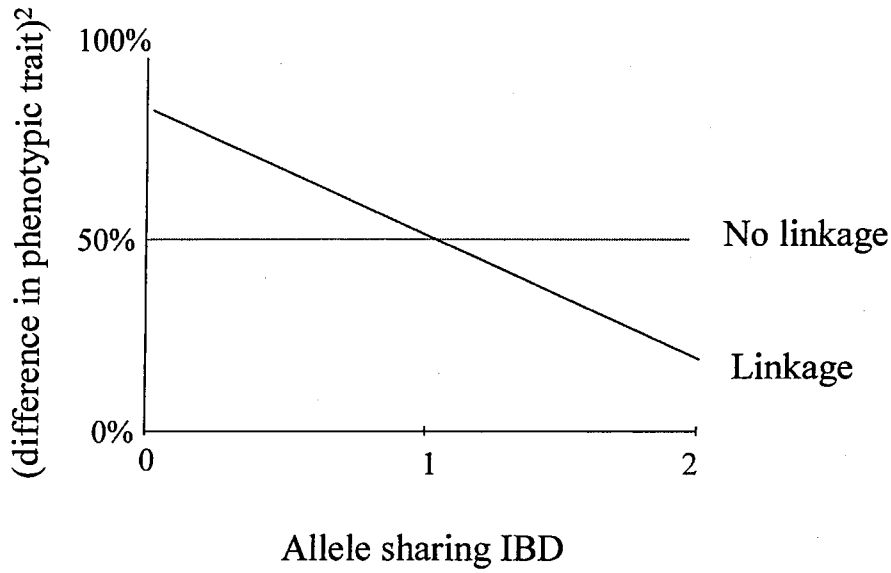
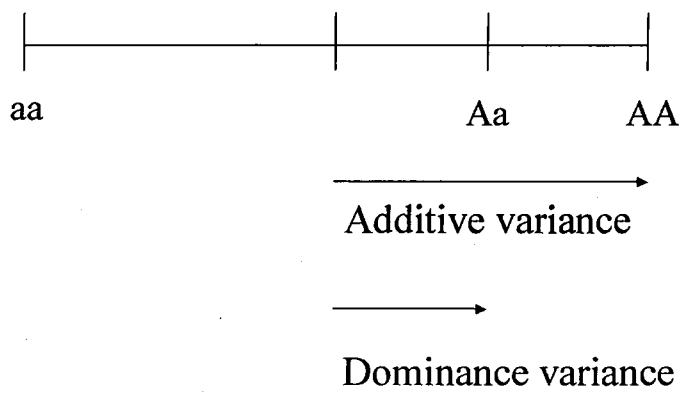


Diagram 1.5.2: Additive and Dominance Variance



Variance component linkage analysis is based upon a biometrical model of inheritance. At its simplest this model consists of a single locus with two alleles A and a, with allele frequencies of p and q = (1-p) respectively. There are three possible genotypes AA, Aa, aa with frequencies of p^2, 2pq and q^2 respectively. The additive genetic value (a) is half the phenotype difference between the phenotypes of the two homozygotes. The dominance genetic value (d) is the distance between the heterozygote phenotype and the midpoint between the two homozygotes (see Diagram 1.5.2).

The mean effect at the locus is given as:

$$\mu = \Sigma f_i x_i = a(p-q) + 2dpq$$

and the total genetic variance at that locus:

$$\sigma^2 = \Sigma f_i(x_i-\mu) = 2pq\,[a+(q-p)d]^2 + [4p^2q^2d^2]$$

where μ = mean trait value

f_i = frequency of the ith genotype

x_i =genetic value of the ith genotype

a = additive variance

d = dominance variance

p = frequency of allele A

q = frequency of allele a = (1-p).

The additive component of the total variance (V_A) is $2pq\,[a+(q-p)d]^2$ and the dominance component (V_D) is $[4p^2q^2d^2]$.

The total genetic variance underlying a complex trait is thus partitioned into the cumulative additive and dominance variance at many loci ($V_G = V_A + V_D$). The total

environmental variance (V_E) can be split into unique environmental effects specific to the individual (V_S) and environmental effects common to the family or population (V_C). Therefore the full model for the variance of a quantitative phenotype is given by:

$$V_T = V_A + V_D + V_E + V_C$$

At a population level, the mean, standard deviation and variance of a quantitative trait like BMD are known. Family data is then used to estimate the different components of the total variance and to give estimates of heritability (as described above).

Variance components linkage analysis is an extension of the above analysis. The estimate for overall heritability can be partitioned into a linked major gene effect and an unlinked remaining genetic effect (potentially due to many genes). The numbers of alleles shared at a locus by a pair of relatives will determine the extent of their covariance. If the locus under consideration affects the quantitative trait, then as the number of alleles shared by the pair increases so their covariance also increases.

$$V_T = V_A + V_D + V_E + V_C + V_{ga} + V_{gd}$$

$$\sigma^2_T = \sigma^2_A + \sigma^2_D + \sigma^2_E + \sigma^2_C + \sigma^2_{ga} + \sigma^2_{gd}$$

$$\text{and } Cov(X_iX_j) = \pi_{ij}\sigma^2_{ga} + \Delta_{ij}\sigma^2_{gd} + \varphi_{ij}\sigma^2_G \qquad \text{if } i \neq j$$

$$= \sigma^2_A + \sigma^2_D + \sigma^2_{ga} + \sigma^2_{gd} \qquad \text{if } i = j$$

where:

X_iX_j are the trait values for pedigree members i or j.

π_{ij} = the proportion of genes shared identical by descent

σ^2_{ga} = major gene additive effect $(2pq[a-d(p-q)]^2$

Δ_{ij} = the probability the pair share both alleles IBD (the only circumstance where dominance can be assessed)

σ^2_{gd} = major gene dominance effect $[4p^2q^2d^2]$.

φ_{ij} = the coefficent of relationship between the pairs of individuals, i.e. the mean probability that they share alleles IBD based on their relationship.

σ^2_G = residual polygenic component

V_{ga} = variance due to a major gene additive effect

V_{gd} = variance due to a major gene dominance effect.

(often the residual environment and polygenic effects are not partitioned).

Maximum likelihood estimates can be obtained for the major gene effects for each locus under investigation, as well as residual polygenic and environmental variance components, assuming multivariate normality. Two likelihoods are compared: (1) the likelihood of a major gene effect, and (2) likelihood of there being no major gene effect; using minus twice the log likelihood ratio to establish significance. As well as identifying a locus, variance components linkage analysis can give a conservative estimate of the magnitude of genetic effect of that locus (Amos et al. 1996). Variance components analysis also explicitly allows for statistical nonindependence of siblings, and thus type 1 errors are less likely (Wijsman et al. 1997).

The variance components for each trait locus, simultaneously with estimates of the residual polygenic variance, are estimated from the data at each chromosomal site. Comparison of variance components analysis and sib-pair based approaches show that

although type 1 error rate is similar, variance components analysis is more powerful and efficient (Amos et al. 1997; Williams et al. 1999). Additionally the use of general pedigrees, rather than nuclear families, also increases statistical power (Williams et al. 1999), although this may depend upon the genetic model for the trait (Badner et al. 1998).

Violation of the assumption of multivariate normality by ascertainment (such as choosing probands with extreme trait values) results in bias of this statistic, although this can be corrected if the ascertainment criteria are known (de Andrade et al. 1997). Failure to correct for ascertainment bias tends to lead to loss of power, rather than to inflation of type 1 error (Fisher et al. 1999; Marlow 2001).

All the different linkage packages, whether model-free or model-based, require that 1) all relationships among pedigree members are accurately known, 2) marker-allele frequencies are accurately known, and 3) all marker-typing data are correct. The impact of genotyping errors upon the ability to detect linkage is considerable. A 1% error rate in genotyping results in the loss of 21-58% of linkage information (Douglas et al. 2000). Thus for loci contributing modestly to the overall susceptibility to disease, genotyping errors necessitate substantial increases in sample size for such loci to be detectable.

1.5.2 Linkage Disequilibrium and Association

Linkage disequilibrium refers to the inheritance of markers together on a chromosome strand ('haplotype') in a population more often than would be expected by chance. When a disease-causing mutation occurs it will be inherited together with the surrounding DNA unless recombination occurs. Recombination is unlikely between the mutation and the

markers lying very close to it, resulting in a haplotype of marker alleles and the mutation. This haplotype will be found more often in disease cases than in healthy individuals; thus the frequency of marker alleles near the mutation will differ between affected and unaffected individuals. This will not of course be observed in diseases where a high proportion of cases are due to new mutations, where multiple disease-associated haplotypes exist or where there are phenocopies.

To become common in the population, the disease-causing mutation must have occurred many generations previously and thus many meioses will have occurred since the original mutational event. Only alleles lying very close to the mutation will remain on the same haplotype in linkage disequilibrium. The extent of linkage disequilibrium varies considerably throughout the genome and in different populations. Populations with relative genetic homogeneity have wider areas of linkage disequilibrium. In some areas of the genome, most notably the major histocompatibility complex, evolutionary pressure has maintained linkage disequilibrium over areas as great as 5-6 cM, whereas on average throughout the rest of the genome it may only be observed over $\leq$1cM (discussed further below). In genes with high mutation rates (e.g. fibrillin) the area of surrounding linkage disequilibrium may be extremely narrow (<1 kb – not even measurable across the coding region of a large gene such as fibrillin). Linkage disequilibrium may be so weak as to be unmeasurable on a population level. Such disease-causing genes are difficult to identify by linkage disequilibrium or association methods (Terwilliger et al. 1998).

Population association studies include case-control studies of unrelated individuals and Analysis of Variance (ANOVA methods). Case-control studies compare the frequency of

particular genetic polymorphisms between cases and controls, whereas ANOVA methods compare the variances within and between each genotype group. A significant result may arise for several reasons. The genetic polymorphism being examined may be the actual disease-causing mutation and therefore more prevalent in the cases. If the polymorphism is a marker in strong linkage disequilibrium with the disease-causing allele then again association will be evident. Spurious association may result if individuals are not drawn from the same population, as the frequencies of marker alleles will differ between cases and controls whether or not the markers are in linkage disequilibrium with the disease-causing mutation. This is referred to as population stratification. The high false-positive rate of case-control association studies is frequently attributed to this cause. However, an alternative reason is that in most cases the prior probability of a particular polymorphism being causally related to the trait under consideration is low (Risch 2000).

1.5.3 Within-Family Association Methods

Within-family association methods were developed to circumvent bias from population stratification in association studies. In essence, allele frequencies are compared between cases and their relatives rather than with unrelated controls. Many types of relatives could be used, but in practice parents have been the most common choice.

Most simply, one could compare the frequency of all marker alleles (both transmitted and non-transmitted) in parents (controls) and the alleles present in an affected child (case). However, power would be lost by effectively double-counting the transmitted alleles in both the case and the control groups. Hence the haplotype relative risk statistic was developed by Rubinstein and Falk (Falk et al. 1987). For this statistic, the sampling unit

consists of a family with two parents and an affected child (the 'case'). Each unit contributes the case genotype (the child) and an artificially constructed 'control' genotype from the two parental alleles not transmitted to the child. The case and control genotypes are regarded as independent samples and are compared in a contingency table as would be used for an unmatched case-control study, with the presence or absence of a specified genotype used as the 'exposure' status. The odds ratio obtained is known as the haplotype relative risk. If the non-transmitted alleles are a random sample from the general population, and if the recombination fraction between the marker and the disease loci is 0, it is a true estimate of the population relative risk.

The haplotype-based haplotype relative risk statistic uses alleles rather than genotype as the unit of observation and compares the proportion of a particular allele transmitted with the proportion not transmitted (Terwilliger et al. 1992). Thus each sampling unit contributes two transmitted and two non-transmitted alleles to the total allele numbers in the case and control groups respectively. These groups are then compared as two independent case-control samples. The test statistic corresponds to McNemar's statistic, which is asymptotically distributed in a chi-squared distribution with 1 degree of freedom. In contrast with the transmission disequilibrium test (discussed below), parents may be homozygous or heterozygous for the marker and still contribute information. Also, the haplotype relative risk statistic can use only one affected child per family. Because it is a comparison of total allele frequencies in the different groups, it is purely a test of association and not of linkage.

Haplotype relative risk methods assume that the transmitted and non-transmitted alleles contributed by a parent are independent observations whereas in fact they are paired data. Transmission disequilibrium testing (TDT) was developed to compare the paired data of the transmitted and non-transmitted alleles from heterozygous parents, testing differential transmission of alleles (Spielman et al. 1996; Spielman et al. 1993). Suppose at a particular marker there are two alleles M1 and M2. In the absence of linkage or association, heterozygous parents should transmit M1 and M2 with equal frequency. However if the M1 allele is in linkage disequilibrium with a disease-associated allele then it will be transmitted more often to affected children. TDT can be extended for highly polymorphic markers rather than the simple two-allele model used in explanation, so that for each heterozygous parent genotype M_iM_j, the number of times M_i is transmitted to affected offspring is compared with the number of times M_j is transmitted (Sham et al. 1995). Distortion in marker allele transmissions from a heterozygous parent to an affected child can only occur if the marker and disease loci are linked.

The advantage of TDT over haplotype-based haplotype relative risk methods is that TDT is concerned with transmission of alleles rather than a comparison of allele frequencies in different patient groups and thus it tests for linkage in the presence of linkage disequilibrium. Most other linkage analyses (e.g. affected sib-pair methods) require more than one affected child per family. TDT only requires a single affected individual and their (heterozygous) parents to be informative for linkage; however in this setting it is a test of linkage disequilibrium in the presence of linkage.

In the case of randomly ascertained singleton cases and their parents, TDT is a test of both linkage and of linkage disequilibrium (Sham et al. 1995). TDT can be performed in multiplex families but in this situation it is valid only as a test of linkage, not of linkage disequilibrium. This is due to the bias introduced by intra-familial linkage disequilibrium between a particular marker allele and the disease locus. For example, consider a large pedigree with multiple affected members all descended from a single affected founder. The ancestral haplotype, containing the marker allele and the disease allele, would be inherited by all the affected individuals (assuming no recombination between marker and disease loci). Greater transmission of the marker allele to the many affected cases from this one pedigree would bias the association data towards this particular marker allele. Thus linkage would (correctly) be observed, but no inference about an association of the disease and marker alleles at a population level could be made (J. Terwilliger, personal communication quoted in (Spielman et al. 1996)). However, the transmission disequilibrium statistic has been extended so that multiplex pedigrees can be used with the statistic remaining a valid test of both linkage and linkage disequilibrium (Martin et al. 1997). In general, TDT is always a measure of linkage with increasing power in the presence of linkage disequilibrium.

TDT requires two heterozygous parents to be informative. If data were included from families in whom only one parental genotype was known, the results would be biased according to the frequency of the most common allele. If the known parent had genotype M_1M_2 and the affected child was of genotype M_1M_1 it would be assumed (correctly) that the known parent had transmitted M_1 to the child. However, if the child were heterozygous with genotype M_1M_2 then the data would be discarded, as no comment

about allele transmission from the known parent could be made. The more frequent M_1 is in the population, however, the more likely it would be the allele transmitted by the unknown parent. Thus data on the transmission from the available M_1 M_2 parent would be used when that parent transmitted M_1 (as the child would be homozygous for M_1) but not used if the parent transmitted M_2 (resulting in a heterozygous genotype), biasing the statistic (Curtis et al. 1995; Dudbridge et al. 2000). The data would also be used if the child had an allele (M_3) not seen in the single known parental genotype, as this obviously came from the other parent (Curtis et al. 1995).

The use of TDT may be valid despite missing parental genotypes if siblings can be used to establish parental genotypes and phase. Several statistical methods to infer missing parental genotypes in an unbiased manner have been developed (Abecasis et al. 2000; Dudbridge et al. 2000; Fulker et al. 1999). Alternatively, TDT may use unaffected siblings as controls instead of parents (Allison et al. 1999; Curtis 1997; Spielman et al. 1998), however greater numbers of families are needed to achieve equivalent power (Curtis 1997).

TDT may be biased by segregation distortion (in which there is preferential transmission of a particular allele to all viable offspring, whether disease-affected or not). Allele transmission to non-affected siblings may be compared with transmission to the affected offspring to demonstrate equal transmission of alleles and to exclude such meiotic drive (Parsian et al. 1991).

TDT may also be used for quantitative data (Allison 1997; Allison et al. 1999) and may be a more powerful test for detecting linkage than Haseman-Elston (1972) or the extreme

sib-pair analysis of Risch and Zhang (1996). As with both of these methods, the use of probands with extreme trait values increases the power of quantitative TDT (QTDT) to detect linkage (Allison 1997).

Abecasis and colleagues recently extended QTDT by using variance components modelling, using nuclear families of any size, with or without parental information. Although missing parental genotypes still reduces power, this loss is negligible in families with four or more genotyped siblings (Abecasis et al. 2000).

There has been considerable debate about the use of linkage disequilibrium methods (such as TDT) in whole genome scanning. Single nucleotide polymorphisms (SNPs) are bi-allelic loci occurring at very high frequency throughout the genome. Recently released SNP maps include over 2.5 million markers genome wide (http://snp.cshl.org/snp and http://www.ncbi.nlm.nih.gov/SNP). Linkage disequilibrium methods may revolutionise detection of disease-causing genes. Traditionally, disease-causing genes have been identified by positional cloning. A relatively large (~10cM) area containing one or more disease-causing genes is identified by linkage in families. Linkage disequilibrium mapping (by TDT, haplotype-based haplotype relative risk statistics or other means) is an efficient way of further fine mapping of the gene(s) of interest (Todd 1995). Candidate genes previously identified by gene expression studies or from a known biological role in the disease that are located within the isolated region can then be examined by mutational screening. An alternative way would be to undertake linkage disequilibrium mapping over the whole genome (initially proposed by Risch and Merikangas (Risch et al. 1996)). This would critically depend upon the extent

of linkage disequilibrium throughout the genome. Using results from simulation studies, Kruglyak argued that useful linkage disequilibrium only extends for an average of 3 kB, and thus many thousands of markers (~ 500 000 SNPs) would be needed for such an undertaking (Kruglyak 1999). However, the simulations did not include provision for natural selection. For disease-causing alleles for common diseases (such as cardiovascular disease) to become sufficiently common in the population there must be selective pressures for these alleles to persist in the population. Several studies have demonstrated that linkage disequilibrium is more extensive than these estimates and in some areas extends as far as 500 kB (Abecasis et al. 2001; Eaves et al. 2000; Moffatt et al. 2000). However, a study of SNPs of the lipoprotein lipase gene showed that there may be no linkage disequilibrium between many SNPs even within this one gene (Clark et al. 1998; Templeton et al. 2000), although a functional genetic effect upon lipoprotein lipase would be likely to have a severe detrimental effect upon survival of the individual and thus there would be selection against a disease-associated haplotype of this gene.

Thus beliefs about the feasibility of linkage disequilibrium mapping using SNPs fall broadly into two camps. On one hand, the number of markers required may be prohibitive (~ 500000), as it is likely that for many genes causing common diseases several mutations may be involved, each with little measurable linkage disequilibrium on a population level. The alternative view is that common disease variants, with broad surrounding linkage disequilibrium, cause common diseases. Therefore the required marker density for detection of such common variants may be as low as one SNP every 1 cM, a total of 3400 for the whole genome. However, the statistical analysis would have to use Bonferroni correction to adjust for multiple independent observations drawn from

the same set of data with substantial effect upon significance levels. Correction of significance levels for SNP mapping will also be much greater than for linkage analysis in order to adjust for each marker's independence and for the number of alleles. Further, if linkage disequilibrium does not extend sufficiently far for a 1 cM map, then disease-causing loci will be missed. Extensive use of linkage disequilibrium mapping using SNPs over the next few years will prove which view is correct.

1.6 A Review of Bone Physiology

A summary of bone physiology is presented to give context to the genetic studies in osteoporosis reviewed in Sections 1.7 and 1.8.

Bone can be divided into cortical bone, the tubular component of long bones, and trabecular bone, the meshwork of interconnected rods and plates of bone making up the core of the tubular bones and the body of the vertebrae. Trabecular bone is the more metabolically active part of bone, due to the extensive surface area available for resorption and reformation. Thus it is the more susceptible part of bone to factors affecting either resorption or bone formation.

Bone cells (osteocytes, osteoclasts and osteoblasts) respond to endocrine, paracrine, autocrine and mechanical signals, resulting in bone formation, resorption, remodelling and repair.

1.6.1 Bone Formation

The cells responsible for bone formation are stromal osteoprogenitor cells (mesenchymal progenitor cells derived from the periosteum and bone marrow); osteoblasts (synthesizing bone matrix on bone forming surfaces); osteocytes (resulting from the further differentiation of osteoblasts engulfed within the structure of the bone matrix) and the supportive bone surface lining cells or endosteum.

Osteoblastogenesis requires bone morphogenetic proteins (BMPs) for uncommitted progenitor cells to differentiate into osteoblasts. BMPs, especially BMP-7, result in transcription of an osteoblast-specific transcription factor, core binding factor a1 (Cbfa-1). Cbfa-1 activates osteoblast-specific genes including osteopontin, bone sialoprotein, type 1 collagen, and osteocalcin. Additionally, BMPs induce a homeobox gene, distal-less 5 (dlx-5), which regulates osteocalcin and alkaline phosphatase expression and the process of mineralization. The crucial role of Cfba-1 in osteoblast development is illustrated by Cfba-1 knockout mice, which completely lack osteoblasts (Otto et al. 1997). Other growth factors can influence committed osteoblast progenitors, notably transforming growth factor β (TGF-β), platelet derived growth factor (PDGF), insulin-like growth factors (IGF-1 and -2) and fibroblast growth factors (FGFs). Parathyroid hormone (PTH), parathyroid hormone-related peptide (PTHrP), glucocorticoids, and prostaglandin E_2 also influence osteoblastogenesis.

Osteoprogenitor cells grow and proliferate before finally differentiating into osteoblasts. Osteoblasts are characterised by biosynthesis and organization of the bone extracellular matrix, secreting both type 1 collagen and bone matrix proteins as osteoid. Accumulation

of extracellular matrix results in down-regulation of osteoblast growth and proliferation. Maturation of the bone matrix follows, with collagen fibril modification and increased osteoblastic secretion of specialised bone matrix proteins, such as osteopontin, osteocalcin and bone sialoprotein. The osteoid is then mineralised by deposition of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) to become bone.

Osteoblasts finally differentiate into osteocytes as the cells are subsumed within mature bone. Osteocytes have numerous extensions of their cell membrane through the canaliculi of bone. Osteocytes communicate with each other and with osteoblasts on the surface of the bone through gap-junction proteins (connexins) connecting the cell types. This results in a cellular syncytium capable of an integrated response to the various stimuli upon the skeleton. Both osteoblasts and osteocytes have receptors for PTH, $1,25(OH)_2D_3$, estrogen, and the many cytokines that influence bone turnover.

1.6.2 Extracellular Matrix Proteins Produced by Osteoblasts

1.6.2.1 Collagen

The predominant protein of bone is type I collagen, making up 90% of its organic makeup. Type I collagen is a triple helical structure, consisting of two α1(I) chains and one α2(I) chain. The chains associate into a left-handed helix, which then twists into a right-handed helix, to form a final structure of a rope-like rod (reviewed in (Lian et al. 1999)).

Each chain consists of about 1000 amino acids (AA) consisting of a gly-X-Y repeating triplet. It is essential that glycine is the third amino acid, as it is the only AA small

enough to fit in the centre of the triple helix. The X position amino acid is frequently proline, the Y position frequently 4-hydroxyproline. The X and Y amino acids limit rotation of the chain. Additionally the side chains of the X and Y amino acids lie externally on the surface of the molecule, and promote polymerisation of the collagen: interaction between clusters of hydrophobic and charged side chains direct self assembly into polymers (Prockop et al. 1995).

The chains are synthesised as large precursor molecules (procollagen). Post-translational modifications include cleavage of signal peptides, hydroxylation of particular proline and lysine residues in position Y, glycosylation of certain lysine and hydroxylysine residues, and formation of intra and inter-molecular covalent di-sulfide cross-links. The carboxy-terminal propeptides associate together forming a nucleus of a triple helix, which conformation is then propagated to the amino-terminal region in a zipper-like fashion. The soluble procollagen molecule is secreted but then undergoes cleavage of both amino- and carboxy-terminal propeptides and conversion of some AA residues to aldehydes. The collagen then self-assembles into tightly packed striated fibrils, with staggering of each molecule by approximately one-quarter its length relative to its neighbour. Further cross-linking renders the collagen insoluble (Prockop et al. 1995).

The cross-links of collagen differ in different connective tissue types. Bone resorption results in release of relatively bone-specific type I collagen cross-links that can be assayed in urine or blood as a measure of bone degradation. Examples include deoxypyridinoline and C- or N- terminal cross-linked telopeptides of type I collagen (Woitge et al. 2000). N- or C- propeptide cleavage products can be assayed as a measure

of collagen formation, with intact procollagen type I aminoterminal propepetide (PINP) the most sensitive marker (Gundberg 2000).

In addition to type 1 collagen, trace amounts of other types of collagen are found at various stages of bone development.

1.6.2.2 Non-collagenous Proteins

Non-collagenous proteins make up 10-15% of the total bone protein content. These include both endogenously- and exogenously-derived proteins. The predominant exogenously-derived proteins are albumin and α_2-HS-glycoprotein, accounting for 25% of the non-collagenous proteins. The endogenous proteins are produced by bone cells on a mole-to-mole basis with type 1 collagen. These include proteoglycans (e.g.biglycan and decorin), glycoproteins (including alkaline phosphatase and osteonectin), glycosylated proteins involved in cell attachment (including fibrillin, osteopontin, bone sialoprotein, fibronectin, vibronectin, thrombospondin and type 1 collagen itself) and finally γ-carboxylic acid (gla)- containing proteins (matrix-gla protein, osteocalcin and protein S).

1.6.2.3 Glycosylated Proteins in Bone

The glycosylated proteins in bone contain an RGD (Arg-Gly-Asn) motif. This consensus sequence then binds to the integrin class of cell surface attachment molecules, although cell attachment may also occur independently of RGD. Additionally osteopontin and bone sialoprotein bind calcium ions by polyacidic amino acid sequences.

Osteopontin is the most abundant phosphorylated glycoprotein of the extracellular matrix of bone and has an important role in bone remodelling. Osteopontin mediates the binding of osteoclasts to the bone matrix through the integrin $\alpha_V\beta_3$ receptor present on the osteoclast membrane (Reinholt et al. 1990) and thus potentiates bone resorption. Additionally osteopontin promotes the adherence of osteoblasts, fibroblasts, macrophages, and T-cells. It is thought that osteopontin directs cellular migration during skeletal growth and remodelling, and affects the degree of mineralization of the extracellular matrix (Gerstenfeld 1999). At least in part this is achieved through the facilitative effects of osteopontin upon angiogenesis. Osteopontin enables efficient vascularisation of bone thus supporting osteoclastogenesis (Asou et al. 2001).

During development, the osteopontin gene is expressed ubiquitously in skeletal tissues but is also present in other tissues including the kidney. In the mature skeleton osteopontin is produced by both osteoblasts and osteoclasts. It is distributed throughout the mineralised part of bone and in hypertrophic cartilage. Osteopontin gene expression in bone is regulated by 1α,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) through a vitamin D response element (Staal et al. 1996). Osteopontin gene expression is also induced in response to mechanical stimulation (Terai et al. 1999).

Osteopontin is also expressed in many other tissues, with important roles in malignant growth and metastatic potential of tumours, mediation of tissue inflammation and repair and as a CD44 ligand (reviewed in (Gerstenfeld 1999)). Osteopontin is a constitutive protein of normal elastic fibres of the aorta and skin. These fibres have a tendency to calcify and osteopontin may regulate their mineralization ((Baccarini-Contri et al. 1994),

quoted in http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?166490). Osteopontin may play a role in urinary stone formation as calcium oxalate stones consist of osteopontin protein (Kohri et al. 1992) and osteopontin mRNA has been demonstrated in the distal tubular cells of stone-forming rats (Kohri et al. 1993).

Bone sialoprotein expression is specific to bone and highly correlated with the appearance of mineralization.

Fibrillin is a large (350 kDa) RGD-containing glycoprotein. In bone, fibrillin is secreted by osteoblasts into the extracellular matrix, where it forms 10-12 nm microfibrils. In adult bone, fibrillin microfibrils are found mainly on the bone surface, especially at the sites of insertion of tendons and ligaments. Although found throughout the bone cortex during development, in adult cortical bone fibrillin is found mainly in the canaliculi and cement lines (Keene et al. 1991). Fibrillin is also found pericellularly to cells lining the endosteal surfaces of trabecular bone, some osteocytes and cells surrounding blood vessels (Kitahama et al. 2000). Fibrillin is constitutively expressed by osteoblasts with expression unaffected by many skeletally active agents (Kitahama et al. 2000).

1.6.2.4 Bone gla Proteins

The bone gla proteins (matrix-gla protein, osteocalcin and protein S) are post-translationally modified by vitamin K-dependent γ-carboxylation, producing dicarboxylic glutamyl residues with enhanced calcium binding. Osteocalcin (also known as bone gla protein) is the most abundant non-collagenous protein of the bone extracellular matrix. Osteocalcin expression occurs almost exclusively in osteoblasts; hence serum osteocalcin

levels have been used as a measure of bone formation. Expression is regulated by a $1,25(OH)_2D_3$ response element in the osteocalcin promoter region (Kerner et al. 1989). The physiological role of osteocalcin in humans is not entirely clear. Its release may mark the turning point between bone formation and resorption. The strong binding of osteocalcin to hydroxyapatite is critically dependent upon its 3 residues of γ carboxyglutamic acid. Osteocalcin may have an inhibitory role for bone mineralization (Lian et al. 1999).

1.6.3 Bone mineralization

Mature bone consists of 50-70% mineral, 20-40% organic matrix, 5-10% water and 3% lipid. Bone mineral provides mechanical rigidity and load bearing strength to the skeleton. It consists of crystals of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) with impurities (such as carbonate, magnesium, strontium and acid phosphate) and vacancies (missing hydroxyl groups) (Glimcher 1998). The imperfections result in increased hydroxyapatite solubility, giving the skeleton an additional role as a buffer against serum changes in calcium and phosphate ion levels.

Crystal growth commences with nucleation – the generation of the first stable crystal from its component ions. Subsequent growth is due to addition of ions and aggregation of ion crystals (Landis 1995). Bone mineral crystals are initially extremely small (less than 200Å) but as bone matures, the hydroxyapatite crystals become larger and more perfect.

The mechanical role of the organic matrix – which is predominantly type I collagen – is to provide elasticity and flexibility. Additionally, both the collagenous and non-collagenous matrix proteins influence bone mineralization, by promoting nucleation and regulating the size, shape and orientation of crystal growth. The proteins affecting mineralization include collagen itself, osteopontin, osteocalcin, biglycan, osteonectin, thrombospondin, bone sialoprotein and matrix gla protein. Several of these proteins are phosphoproteins. Enzymes that regulate the phosphorylation and dephosphorylation of these proteins (such as alkaline phosphatase) also affect bone mineralization (Lian et al. 1999).

1.6.4 Bone Remodelling

Bone undergoes continuous regeneration both during development and growth and in the mature adult skeleton. In the growing skeleton, modelling results in increasing trabecular thickness and widening of the marrow cavity of long bones. Remodelling in the adult skeleton replaces old bone with new, most probably to repair fatigue fractures, to prevent accumulation of old bone, and in response to physical stress. Remodelling affects trabecular bone disproportionately, such that 25% of trabecular bone undergoes remodelling annually, compared with 3% of cortical bone (Manolagas et al. 1995).

Remodelling requires close temporal and spatial coupling of osteoclasts and osteoblasts so that bone resorption and formation are closely matched. The basic multicellular unit (BMU) or bone modelling unit consists of the collection of osteoclasts and osteoblasts with supporting vascular, neural and connective tissue structures needed for the orderly remodelling of bone. Multicellular osteoclasts at the front resorb bone by acidification

and proteolytic digestion at their ruffled cell membrane border. The osteoclasts then undergo apoptosis and are replaced by osteoblasts that cover the bone surface and secrete osteoid. Osteoid is then mineralised to form new bone. In cortical bone the BMU moves in a tunnel through the bone. In trabecular bone the BMU moves in a trench across the trabecular surface (Manolagas 2000).

The close relationship between osteoclast and osteoblast formation and activation is achieved by a complex system involving cytokines and growth factors, systemic hormones, and cell-adhesion molecules such as matrix proteins. Cytokine production in bone is notable for its extensive redundancy (discussed below) and complex positive and negative feed back loops with both autocrine and paracrine regulation (also discussed below). Most systemic hormones exert their effect on osteoblastogenesis and osteoclastogenesis indirectly, through local cytokine production (Manolagas et al. 1995). Many of the same factors are capable of influencing both osteoblastogenesis and osteoclastogenesis, thus maintaining their functional and temporal relationships.

Cbfa-1 knockout mice illustrate the tight coupling of osteoblasts and osteoclasts. Abrogation of the Cbfa-binding site in the RANKL gene promoter region blocks both osteoblastogenesis and osteoclastogenesis (Otto et al. 1997). Additionally other mice strains with defective osteoblastogenesis exhibit decreased osteoclastogenesis (Jilka et al. 1996; Weinstein et al. 1997).

1.6.4.1 Osteoclast Formation: the OPG/RANKL/RANK Axis

Remodelling commences with osteoclast activation and formation, followed by bone resorption and finally osteoclast apoptosis. Osteoclastic precursors come from the haematopoietic cells of the macrophage/monocyte lineage (Hattersley et al. 1991). Osteoclastogenesis is induced by both many cytokines (including IL-1, IL-6, TNF, M-CSF) and by systemic hormones $1,25(OH)_2D_3$ and PTH. However, a major axis in osteoclastogenesis has recently been described, involving TNF-related proteins RANKL (Receptor Activator of NF-κB ligand), RANK (receptor activator of NF-κB) and Osteoprotegerin (OPG). A number of synonyms for these three proteins exist (Table 1.6.4.1).

Table 1.6.4.1 Synonyms for OPG, RANK and RANKL (Riggs et al. 2000)

Accepted term	Synonyms
RANKL	Receptor activator of NF-κB ligand Osteoclast differentiation factor (ODF) TNF-related activation-induced cytokine (TRANCE) Osteoprotegerin ligand (OPGL) Stromal osteoclast-forming activity (SOFA) TNF superfamily 11
RANK	Receptor activator of NF-κB Osteoclast differentiation and activation receptor (ODAR) TNF superfamily receptor 11A
Osteoprotegerin (OPG)	Osteoclastogenesis inhibitory factor (OCIF) TNF receptor related molecule 1 (TR1) Follicular dendritic receptor 1 TNF superfamily receptor 11B

RANKL is a TNF-related cytokine critical for osteoclastogenesis. It is a polypeptide of 317 amino acids and exists in both membrane bound (40-45 kDa) and soluble (31 kDa) forms (Lacey et al. 1998; Yasuda et al. 1998). Its gene has been localised to chromosome 13q14 (Wong et al. 1997) and contains a response element for Cbfa-1 (O'Brien et al. 1998). Cbfa-deficient mice (as mentioned above) are deficient in RANKL mRNA expression and osteoclastogenesis (Gao et al. 1998).

Expression of RANKL is found in many tissues but is highest in committed pre-osteoblastic cells and T lymphocytes. RANKL mRNA levels in osteoblasts are up-regulated by many factors that stimulate bone resorption including DXAmethasone, IL-1, IL-6, IL-11, TNF-α, PTH, $1,25(OH)_2D_3$, and prostaglandin E_2. Conversely, RANKL

mRNA levels are down-regulated by TGF–β which suppresses bone resorption. Administration of either OPG (see below) or anti-RANKL antibodies results in blocking the bone resorption induced by $1,25(OH)_2D_3$, PTH, IL-1, and PGE_2 (Takai et al. 1998; Tsukii et al. 1998).

RANKL, in the presence of M-CSF and in the appropriate bone stromal cell environment, is both necessary and sufficient for osteoclast formation, maturation and activation (Burgess et al. 1999; Fuller et al. 1998). RANKL stimulates osteoclast differentiation from pluripotent osteoclast precursors through to mature multinucleated osteoclasts. It enhances the activity of mature osteoclasts, stimulating bone resorption and resulting in osteoclastic pit formation and calcium release (Udagawa et al. 1999). RANKL also prevents osteoclast apoptosis. RANKL exists in both soluble and membrane bound forms, the latter resulting in efficient cell-to-cell signalling with stromal cells (Nakashima et al. 2000).

RANKL knockout mice have severe osteopetrosis, skeletal malformation (short club-like bones), impaired tooth eruption, no mature osteoclasts, and (due to bone marrow cavity narrowing) extramedullary haematopoiesis (Kong et al. 1999). Additionally such mice have defects in B- and T-lymphocyte maturation and abnormal lymphoid tissue development.

RANKL binds with high specificity and affinity to receptor activator of NF-κB (RANK), a transmembrane-bound receptor expressed on osteoclasts and pre-osteoclast progenitors, resulting in osteoclastogenesis and bone resorption (Hsu et al. 1999). RANK is a 616 AA peptide receptor, a member of the TNF receptor superfamily, found on osteoclasts, T

cells and dendritic cells. Its essential role in osteoclastogenesis is demonstrated by transgenic RANK-knockout mice, which develop osteopetrosis (Dougall et al. 1999). Expression of a soluble RANK-Fc fusion protein in normal mice results in decreased osteoclastogenesis, decreased bone resorption and osteopetrosis, due to interruption of osteoblast signalling to osteoclast precursors (Hsu et al. 1999). Physiologically, this signalling is interrupted by osteoprotegerin.

Osteoprotegerin is a soluble 'decoy'-receptor for RANKL, blocking RANKL-RANK interaction. It is a member of the TNF receptor superfamily, unique in that it lacks transmembrane and cytoplasmic domains. Instead it is secreted as a disulfide-linked homodimeric glycoprotein, each monomer consisting of 380AA (Simonet et al. 1997; Yasuda et al. 1998). OPG mRNA expression is found in many bone cells but particularly osteoblasts. OPG mRNA is also found in endothelial cells, aortic smooth muscle cells, fibroblasts, and a wide variety of other tissues including haematopoietic and lymphoid tissues.

OPG binds with RANKL, preventing its binding with RANK. OPG inhibits osteoclast differentiation, suppresses osteoclast activation, prevents osteoclastic pit formation, and induces osteoclast apoptosis (Simonet et al. 1997). OPG also prevents osteoclastogenesis and bony resorption induced by $1,25(OH)_2D_3$, PTH, IL-1, IL-11 and PGE_2 (Hofbauer et al. 1998).

OPG knockout mice have severe osteoporosis with destruction of the femoral growth plates, multiple fractures, low bone mineral density, reduced strength and stiffness of bone, and loss of both cancellous and cortical bone (with near total loss of cancellous

bone by 2 months), a phenotype resembling human osteoporosis (Bucay et al. 1998; Mizuno et al. 1998). Additionally, such mice have vascular calcification affecting particularly the aorta and renal arteries (Bucay et al. 1998) (of note given the clinical observation of the frequency of both osteoporosis and aortic calcification in women (Min et al. 2000)).

OPG overexpression results in severe osteopetrosis with narrowed bone cavities and compensatory extramedullary haematopoiesis (Simonet et al. 1997). Trabecular bone in particular is increased. In contrast to RANKL knockout mice, dentition and immune defects are lacking.

Table 1.6.4.2 Bone Phenotype in Transgenic and Knockout Mice

Osteopetrotic mice	RANK (-/-)(Dougall et al. 1999) RANKL (-/-)(Kong et al. 1999) OPG-Tg (Simonet et al. 1997) RANK:Fc-Tg (Hsu et al. 1999)
Osteoporotic mice	OPG (-/-)(Bucay et al. 1998) RANKL-Tg (Lacey et al. 1998)

Multiple cytokines, growth factors, and systemic hormones affect mRNA levels of OPG and RANKL (see Table 1.6.4.3). The co-stimulation of both OPG and RANKL by many of these factors appears paradoxical. It may be the resulting ratio of expression of the two genes that determines the extent to which remodelling proceeds (Hofbauer et al. 2000).

In addition to these factors, OPG and TGF-β mRNA levels in osteoblasts increase markedly after tensional force is applied to bone, with resultant loss of osteoclasts (Kobayashi et al. 2000). Thus OPG may also be involved in the compensatory response of bone to stress.

Table 1.6.4.3:

Regulatory Effects of Calcitropic Hormones and Cytokines on RANKL and OPG Expression

Adapted from (Hofbauer et al. 2000)

Mediator	RANKL	OPG
$1,25(OH)_2D_3$	Up	Up
IL-1β	Up	Up
IL-1α		Up
TNF-α	Up	Up
IL-6	Up	
TGF-β		Up
Glucocorticoids	Up	Down
Prostaglandin E2	Up	Down
PTH	Up	
IL-11	Up	
TGF-β	Down	Up
17β-Estrodiol		Up
BMP-2		Up

References (Hofbauer et al. 1998; Takai et al. 1998; Vidal et al. 1998).

The OPG/RANKL/RANK axis appears to be a final common pathway for many of the cytokines and hormones affecting osteoclastogenesis (see Table 1.6.4.3) (Hofbauer et al.

2000). Whether it is the sole final pathway is uncertain. On one hand it seems intuitively unlikely given the extensive redundancy in the cytokine network. However, the severe phenotypic effects of OPG-, RANKL- and RANK- knockout mice contrast sharply with the lack of a skeletally abnormal phenotype in IL-1, IL-6 and TNF knockout mice (see below).

1.6.4.2 Interleukin-1, Tumour Necrosis Factor –alpha and –beta and Interleukin-6

The interleukin-1 (IL-1) family includes IL-1α, IL-1β and the IL-1 receptor antagonist (IL-1RA). IL-1α and -β are 17kDa proteins synthesised by a wide variety of cell types, including activated monocytes and osteoblasts. IL-1α and -β are structurally distinct: IL-1α is the acidic form and IL-1β is the neutral form. However both are potent stimulators of bone resorption and are pro-inflammatory cytokines, exerting their effect through stimulation of the IL-1 receptor (see below) (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?147760). In contrast, IL-1RA inhibits the effects of IL-1α and IL-1β by competitive binding to the IL-1 receptor without eliciting any stimulation (Eisenberg et al. 1990), blocking the effects of both IL-1α and -β on bone resorption (Seckinger et al. 1990). It is the ratio between the agonists IL-1α and -β to the antagonist IL-1RA that determines the overall biological effects of IL-1.

TNF-α (cachexin) and TNF-β (lymphotoxin) are released from activated macrophages and mitogen-activated T- lymphocytes respectively. TNF-α and -β are powerful stimuli of bone resorption and decreased collagen synthesis (Bertolini et al. 1986).

Interleukin 6 (IL-6) is expressed and secreted by osteoblasts and stromal cells in response to both cytokines (especially IL-1 and TNF-α) and systemic calcitropic hormones (PTH and $1,25(OH)_2D_3$) (Manolagas 2000). The osteoblast is the most prodigious source of IL-6 known (Mundy 1999). IL-6 acts through a cytokine-specific receptor IL-6R. Binding of IL-6 to its cellular receptor results in activation of the signal transducing protein gp130, which ultimately results in effects upon gene transcription. IL-11, another pro-osteoclastic cytokine, also signals through gp130 after binding with its specific receptor (Kishimoto et al. 1995; Manolagas 2000; Yin et al. 1993). The IL-6R α–subunit also exists in soluble form. The IL-6R α–subunit can bind to soluble IL-6, and then interact directly with membrane-associated gp130 to stimulate intracellular signalling (Tamura et al. 1993).

TNF, IL-1 and IL-6 are involved in mediating the effects of estrogen deficiency in bone (Jilka et al. 1992; Kimble et al. 1996; Pacifici 1996) (see below). Their relative physiological roles in a sex-hormone replete state is arguable, as mice lacking the ability to synthesize, or respond to, IL-1 or TNF do not have an abnormal skeletal phenotype (Ammann et al. 1997; Lorenzo et al. 1998) whilst IL-6 knockout mice have increased bone turnover relative to normal mice but no other abnormality (see below) (Poli et al. 1994).

IL-1, TNF, PTH and $1,25(OH)_2D_3$ stimulate osteoclasts indirectly by stimulating osteoblastic production of cytokines including RANKL (as discussed above), IL-6, IL-11, M-CSF and GM-CSF (Kobayashi et al. 2000; Mundy 1999; Pfeilschifter et al. 1989; Suda et al. 1995; Thomson et al. 1986; Thomson et al. 1987). These factors exert a direct

effect on osteoclasts, promoting proliferation, differentiation, activation and inhibiting apoptosis. IL-6 also has indirect effects upon osteoclastogenesis as IL-6 can independently stimulate RANKL secretion from osteoblasts. IL-1, TNF, PTH and $1,25(OH)_2D_3$ also stimulate production of cell-adhesion molecules that may further promote osteoclastogenesis (reviewed in (Jilka 1998; Manolagas 2000)).

IL-1, IL6 and TNF each have a positive autocrine effect upon their own production (Jilka 1998). Further, there is marked synergy between them such that TNF and IL-1 synergistically promote IL-6 and TNF production, and TNF and PTH also synergistically increase IL-6 (Passeri et al. 1994).

IL-6 plays a pivotal role in mediating bone loss associated with gonadal deficiency. In ovariectomised mice, osteoclastogenesis was blocked by administration of 17-βestradiol or IL-6 neutralising antibody, and IL-6 levels were increased in the culture media of bone marrow cells from the ovariectomized animals (Jilka et al. 1992). Similarly, orchidectomized mice have increased osteoclastogenesis that is blocked by administration of androgen therapy or administration of an IL-6 neutralizing antibody (Bellido et al. 1995).

Estrogen suppresses production of IL-6 through inhibition of TNF- and IL-1-stimulated IL-6 synthesis (Girasole et al. 1992) and (perhaps also) blockade of IL-6 autocrine stimulation, by binding of the estrogen-receptor complex to transcription factors required for activation of the IL-6 promoter, preventing their activity (Stein et al. 1995). Estrogen also blocks production of gp130 and gp80 of the IL-6 receptor on osteoblast and stromal cells (Lin et al. 1997).

IL-6 secretion by mononuclear cells is raised in women after the menopause (Fagiolo et al. 1993; Kania et al. 1995). However, levels of IL-6 are not increased in postmenopausal women with osteoporosis compared with normals (Khosla et al. 1994), and elevated levels of IL-6 have not been found to correlate with BMD or with indices of bone turnover (Kania et al. 1995). However, it should be noted that serum levels of cytokines might not reflect the bone environment. IL-6 administration to estrogen-replete mice stimulates colony forming unit-granulocyte/monocyte proliferation but does not increase osteoclastogenesis or bone remodelling unless IL-6R is also given (Taguchi et al. 1998; Tamura et al. 1993; Udagawa et al. 1995). Thus changes in IL-6 alone are not sufficient to account for postmenopausal bone loss and the effects of estrogen deficiency in humans may be mediated by IL-6R also. In support of this statement, surgical menopause in humans results in an increase in levels of soluble IL-6R. This increase is suppressed by administration of either estrogen or alendronate (Girasole et al. 1995). Serum levels of IL-6R have been correlated with bone resorption markers and with lumbar BMD in postmenopausal women (Chen et al. 1995).

Estrogen does not affect IL-1 secretion but does suppress TNF-secretion, by inhibition of transcription factor binding to the TNF promoter (Kimble et al. 1997).

Ovariectomy in mice lacking biological activity of any one of IL-1, IL6 and TNF does not increase osteoclastogenesis and bone remodelling (Ammann et al. 1997; Lorenzo et al. 1998; Poli et al. 1994). Thus, despite their apparent redundancy in a sex-hormone replete state, the synergistic effects of these cytokines are needed to mediate the effects of estrogen deficiency *in vivo*.

1.6.5 Gonadal Hormones in Bone

The importance of estrogen and the estrogen receptor in control of bone density in women is clear from the rapid bone loss at menopause and the efficacy of hormone replacement therapy. The important role of estrogen in bone metabolism in men also was supported by the report of a male, homozygous for a coding mutation in exon 2 of the estrogen receptor (introducing a premature stop codon) who was tall with unfused bones and had profound osteoporosis (Smith et al. 1995).

As discussed above, estrogen deficiency results in increased bone remodelling, osteoclastogenesis and bone loss. Specifically, estrogen deficiency results in increased osteoclast progenitor numbers, increased production of cytokines promoting osteoclastogenesis, and increased numbers and activity of stromal cells supporting osteoclastogenesis. Estrogen deficiency also increases osteoblastogenesis, which given the close coupling of the two is not surprising. The effects of estrogen are mediated through IL-1, TNF and IL-6 as discussed above.

Androgen deficiency also increases bone remodelling, osteoclastogenesis and bone loss. Androgen deficiency is similarly mediated through cytokines especially upon IL-6 (Girasole et al. 1992).

The differing contributions of testosterone and estrogen upon the skeleton in both men and women have been controversial. Hypogonadism is a common cause of osteoporosis in males, accounting for 30% of cases (Orwoll 1998). Testosterone may be aromatised to estrogen, either systemically or locally (osteoblasts can aromatise androgens to estrogens

(Burich et al. 1992)), and therefore it is not clear that it is testosterone deficiency *per se* that causes low bone mass. Both male and female patients with aromatase deficiency (and thus inability to synthesise estrogen) have osteopaenia, which responds to administration of estrogen (Carani et al. 1997). A recent study in normal elderly men with pharmacologically induced hypogonadism and aromatisation blockade demonstrated that estrogen is the major hormone preventing bone loss (with a small contribution from testosterone), and that both estrogen and testosterone contribute to bone formation (Falahati et al. 2000). The role of testosterone in the skeleton in women is demonstrated by patients affected by the androgen insensitivity syndrome, who have severe osteopaenia even when well-replaced with estrogen (Marcus et al. 2000). Thus both estrogen and testosterone are important in normal skeletal development and maintenance in both men and women.

Serum levels of free estradiol and testosterone correlate with bone mineral density in both sexes, although after multivariate analysis only free estradiol levels remain a significant independent predictor (Greendale et al. 1997; Khosla et al. 1998).

Both androgens and estrogens bind to intracellular sex steroid receptors. Once activated by ligand binding, the receptors interact with a specific response DNA sequence (known as hormone response elements) or with specific transcription factors, such as AP-1 and NF-κB. Additionally, the nuclear matrix structure may affect nuclear binding of steroid receptors and regulation of gene transcription (reviewed in (Waters et al. 1999)).

Two estrogen receptors are known in humans, estrogen receptor–alpha (ER-α) and –beta (ER-β). Both ER-α and ER-β are expressed in human bone on osteoblasts, osteoclasts

and osteocytes (Eriksen et al. 1988; Hoyland et al. 1997; Vidal et al. 1999). These are discussed further in Chapter 4.

1.6.6 Calcitropic hormones

1.6.6.1 Parathyroid Hormone (PTH)

Normal calcium ion homeostasis is under the influence of parathyroid hormone (PTH), $1,25(OH)_2D_3$ and calcitonin. PTH is the most important of these regulators and is secreted from the parathyroid glands as an 84 AA peptide. Its synthesis and secretion are critically determined by the extracellular calcium concentration, monitored by the calcium sensing receptor of the parathyroid glands. In the presence of hypocalcaemia, PTH is secreted to act primarily upon kidney and bone to restore blood calcium levels by both direct and indirect effects.

PTH secretion is critically determined by serum calcium levels. Hypocalcaemia (detected by the surface membrane calcium sensing receptor, see below) results in rapid secretion of preformed PTH. The steep inverse relationship between serum levels of calcium and PTH release results in close control of serum calcium levels, maintaining near constancy. Additionally, serum calcium levels affect rates of intracellular PTH degradation. In addition to the immediacy of PTH secretion, changing calcium levels also have a more sustained effect by regulating PTH gene expression. Intracellular PTH mRNA levels are increased by hypocalcaemia and hyperphosphataemia, and reduced by hypercalcaemia and hypophosphataemia. $1,25(OH)_2D_3$ also affects PTH secretion, PTH

gene expression, calcium sensing receptor gene expression, and parathyroid cellular proliferation (reviewed in (Jüppner et al. 1999)).

In the kidney, PTH acts directly to increase tubular resorption of calcium. PTH also stimulates 1α-hydroxylase to increase $1,25(OH)_2D_3$ levels, resulting in increased intestinal absorption of calcium. PTH also affects phosphate levels by inhibiting tubular phosphate resorption through reduction in expression of the sodium-dependent co-transporter Npt2.

In bone, PTH exerts its effects mainly through osteoblasts; although osteoclast precursors have PTH receptors, these are not present on mature osteoclasts. Hence PTH action upon mature osteoclasts is indirect and due to PTH-stimulated osteoblastic secretion of RANKL, resulting in osteoclastogenesis and osteoclast stimulation (Yasuda et al. 1998). PTH stimulation of RANKL may be direct or may require cytokine intermediaries.

In general, PTH results in rapid release of calcium from the bone matrix. Continuous high-dose administration of PTH causes predominantly osteoclastic resorption and hypercalcaemia (as happens with primary hyperparathyroidism). Lower intermittent doses of PTH can elicit anabolic effects on bone, through induction of proliferation and differentiation of osteoprogenitor cells (Nishida et al. 1994).

PTH secretion follows a circadian rhythm. Some authors have found alterations in this rhythm in postmenopausal women with osteoporosis, compared with postmenopausal women without osteoporosis and premenopausal women. The normal circadian pattern of PTH is of a nocturnal increase in PTH and phosphate; however in postmenopausal

women with osteoporosis, PTH and phosphate levels fail to rise and PTH in fact falls (Fraser et al. 1998). Consistent with this is evidence that urinary fractional excretion of calcium normally falls at night; however, in women with postmenopausal osteoporosis, nocturnal urinary fractional excretion of calcium is unchanged (Eastell et al. 1992). However whether these events are in any way causative or merely responsive to excessive bone turnover in postmenopausal osteoporosis has not been established.

1.6.6.2 PTH-related Peptide (PTHrP)

PTHrP was initially identified as the humoral mediator of hypercalcaemia of malignancy (Wysolmerski et al. 1994). Its role in normal skeletal development has now also been elucidated, with a critical role in foetal skeletal development demonstrated. This is discussed further in Chapter 5.

Its physiological role in adults is still to be fully established, however it has a wide range of biological actions. In contrast to the systemic effect of PTH, PTHrP has a paracrine effect on local tissue functions. The expression of different isoforms of PTHrP may mediate its varied effects in different tissues.

Of note, PTHrP is involved in the calcium homeostasis of lactation. Glandular epithelial cells and myoepithelial cells of the lactating breast produce large amounts of PTHrP, resulting in elevated serum levels in the nursing mother with large quantities being secreted into breast milk. PTHrP is also expressed in the epidermis and hair follicles, the placenta, the parathyroid gland, pancreatic islet and pituitary and in the central nervous system (Strewler 2000).

Both PTH and PTHrP bind to the Parathyroid Hormone Receptor type1 (PTHR1). The physiology of PTHR1 is discussed extensively in Chapter 5.

1.6.6.3 Vitamin D

$1,25(OH)_2D_3$ is the active form of vitamin D. Vitamin D_3 is formed in the skin from ultraviolet B radiation of 7-dehydrocholesterol. The main dietary sources are fatty fish oils and fortified dairy and cereal products. Vitamin D_3 is metabolised in the liver (to $25(OH)D_3$) and then in the kidney (to $1,25(OH)_2D_3$). PTH enhances renal hydroxylation of $25(OH)D_3$ to form the active hormone. $1,25(OH)_2D_3$ negatively regulates renal 1α-hydroxylase and suppresses synthesis and secretion of PTH, thus forming negative feedback loops on its production.

Free $1,25(OH)_2D_3$ enters target cells to bind with a nuclear vitamin D receptor (VDR). The $1,25(OH)_2D_3$-VDR complex forms a heterodimer with the retinoic acid X receptor (RXR). The heterodimer binds to vitamin D response elements (VDRE) within DNA to affect transcription of vitamin D-responsive genes such as osteocalcin, osteopontin and alkaline phosphatase (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?601769). $1,25(OH)_2D_3$ also regulates transcription of PTHR1, PTH and PTHrP. Administration of $1,25(OH)_2D_3$ results in differential effects upon PTHR1 expression according to cell type, with downregulation of PTHR1 expression in osteoblasts and preosteoblasts, but no change of PTHR1 expression in chondrocytes (Amizuka et al. 1999).

$1,25(OH)_2D_3$ increases serum calcium and phosphate levels, by stimulating intestinal calcium and phosphate absorption, renal calcium and phosphate resorption, and bone resorption of calcium and phosphate.

The primary effect of $1,25(OH)_2D_3$ in bone is to mobilize calcium. $1,25(OH)_2D_3$ elicits a paracrine response from osteoblasts (RANKL and other cytokines) to activate osteoclastogenesis and thus bone resorption (see above). $1,25(OH)_2D_3$ also has direct effects upon osteoblasts, such as regulating expression of various osteoblast-specific proteins (e.g. osteocalcin).

$1,25(OH)_2D_3$ paradoxically also promotes bone mineralization. However this is an indirect rather than active process, primarily achieved by maintaining a sufficiently high ion product to allow mineralization to proceed. The bone abnormalities of both VDR homozygous knockout mice and humans with Vitamin-D dependent rickets can be substantially improved if not normalised by dietary maintenance of adequate serum calcium and phosphate levels (Liberman et al. 1999).

1.6.6.4 Calcitonin

Calcitonin is a 32 AA peptide secreted from the parafollicular C-cells of the thyroid. In contrast to PTH and $1,25(OH)_2D_3$, the major biological effect of calcitonin is to inhibit osteoclastic bone resorption (Deftos et al. 1999). Serum calcium levels regulate calcitonin secretion such that acute increases or decreases in serum calcium result in stimulation or suppression of secretion respectively. The effects of chronically abnormal calcium levels upon calcitonin secretion are less clear (Deftos et al. 1999). Nasal

calcitonin administration increases LS BMD in postmenopausal women (Reginster et al. 1995).

1.6.7 Other Cytokines in Bone

Many cytokines influence bone modelling. The following cytokines are not intended to be an exhaustive list but rather are included both because of their relative importance in bone modelling and because they were used in the candidate gene linkage study presented in Chapter 4.

1.6.7.1 Transforming growth factor-beta

Transforming growth factor-beta (TGF-β) is an important regulator of bone formation and resorption. TGF-β exists abundantly in the bone matrix as three isoforms (β1, 2, and 3) of which TGF-β1 is the dominant form (Pfeilschifter et al. 1998). Levels of TGF-β1 and -β2 in bone tissue correlate with histomorphic indices of bone formation and resorption, and with serum markers osteocalcin and bone-specific alkaline phosphatase (Pfeilschifter et al. 1998).

TGF-β1 is produced by a number of bone cells including osteoblasts and bone marrow cells. It is secreted as a propeptide of 390 AA and incorporated into the bone matrix during its formation. TGF-β1 is activated in the presence of plasmin or strongly acidic or alkaline environments, with cleavage of the propeptide to a 25 kDa protein of 112 AA. During bone resorption TGF-β1 is released from the bone matrix and activated by the low pH under the osteoclast ruffled border. TGF-β1 inhibits mature osteoclasts and

osteoclastogenesis, promotes osteoclast apoptosis (Jilka 1998), stimulates osteoblastogenesis and increases osteoblast synthesis of the extracellular bone matrix (Centrella et al. 1994). As discussed above, TGF-β1 increases OPG secretion (Takai et al. 1998), and its action upon osteoclasts may be a secondary phenomenon, mediated through the OPG/RANK/RANKL signalling system.

1.6.7.2 Colony Stimulating Factors 1 and 2: Monocyte/Macrophage Colony Stimulating Factor (M-CSF) and Granulocyte/Monocyte-Colony Stimulating Factor (GM-CSF)

The cytokine colony stimulating factor-1 is critical for osteoclastogenesis and osteoclast function. As mentioned above, M-CSF with RANKL are both essential and sufficient for osteoclast formation, maturation and activation (Burgess et al. 1999; Fuller et al. 1998).

Osteoblasts synthesize CSF-1 as both a membrane bound and a secreted form, a small portion of which is incorporated into the bone matrix. The different molecular forms may be responsible for different localised effects. CSF-1 has a direct effect upon osteoclasts to stimulate proliferation and differentiation and to prevent apoptosis (Jilka 1998).

CSF-2 (GM-CSF) is also synthesised by osteoblasts and has direct effects upon osteoclasts, stimulating proliferation and preventing apoptosis (Jilka 1998).

1.6.7.3 Insulin-like Growth Factors (IGF-1 and -2)

The insulin-like growth factors IGF-1 and IGF-2 are polypeptides of molecular mass 7.6 kDa. The genes coding for IGF-1 and -2 are found on chromosomes 12 and 11 respectively and show marked structural homology with each other and with the insulin gene, suggesting an evolutionary relationship (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?147440). IGF-1 and -2 are present both in the systemic circulation and are synthesized in bone as local regulators. IGF-1 is synthesized in the liver in response to growth hormone (GH) and is carried in the circulation by IGF-binding proteins (IGFBPs), especially IGFBP-3. Peripheral production of IGF-1 however is less GH-dependent (Duncan et al. 1999). In bone, IGF-1 synthesis is stimulated particularly by PTH and other agents that increase cAMP levels and only modestly by GH (Delany et al. 1994), whereas glucocorticoids inhibit skeletal IGF-1 production. Local IGF production is also under the influence of other skeletal growth factors (such as fibroblast growth factors) and local production of IGFBPs.

IGF-1 is strongly associated with linear growth and with acquisition of peak bone mass and bone mineral content. IGF-2 is much more active prenatally compared with IGF-1. Much less is known about the postnatal effects of IGF-2 on bone mass and skeletal growth (Duncan et al. 1999).

IGF-1 enhances collagen and matrix synthesis, inhibits collagen degradation and stimulates osteoblastogenesis (reviewed in (Lian et al. 1999)). IGF infusions in humans increase bone remodelling (Ebeling et al. 1993). Serum levels of IGF-1 are highest in men and women at the time of attaining peak bone mass, and subsequently decline with

age (Goodman-Gruen et al. 1997). IGF-1 content in bone also declines with age in both men and women (Boonen et al. 1997). Serum IGF-1 levels have been shown in some but not all studies to correlate with BMD (reviewed in (Barrett-Connor et al. 1998)). Men with osteoporosis have been reported to have reduced IGF-1 serum levels (Kurland et al. 1997).

1.6.7.4 Epidermal Growth Factor (EGF)

EGF is a potent mitogen for cells of ectodermal and mesodermal origin. EGF is a single-chain polypeptide of 53 AA with MW 6000. It is an analogue of transforming growth factor alpha, sharing 40% homology (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?131530).

In vitro, osteoblast precursors express EGF-receptors. However differentiation into mature osteoblasts results in loss of EGF-receptors (Chien et al. 2000). In vitro, EGF transforms osteoblast-like cells from a polygonal shape to a spindle form, suppresses osteoblast alkaline phosphatase activity, and selectively decreases type I collagen production with alteration of its hydroxyproline content resulting in a more immature fibril (Hata et al. 1984; Kumegawa et al. 1983).

1.6.7.5 Interleukin-4 (IL-4)

IL-4 is a T helper cell derived cytokine initially identified as a B-cell mitogen (Yokota et al. 1986). As well as having potent anti-inflammatory activity, IL-4 prevents bone resorption. IL-4 inhibits osteoclastogenesis, osteoclast function and osteoclast survival (Bizzarri et al. 1994; Riancho et al. 1993; Shioi et al. 1991). IL-4 protects against bone

matrix degradation and (at least *in vitro*) promotes bone matrix mineralization (Ueno et al. 1992). IL-4 inhibits the synthesis of many pro-resorptive and pro-inflammatory cytokines, including IL-1α, TNF-α, and IL-6, and increases synthesis of cytokine inhibitors (such as IL-1-receptor antagonist) (Joosten et al. 1999). IL-4 administration in murine models of rheumatoid arthritis (collagen-induced arthritis) protects against cartilage and bone destruction and suppresses RANKL production (Joosten et al. 1999; Lubberts et al. 2000).

1.6.7.6 Interleukin-11 (IL-11)

Interleukin 11 is an osteoblast/stromal-cell derived lymphopoietic and haematopoietic cytokine which stimulates osteoclastogenesis and bone resorption. The biological similarities between IL-11 and IL-6 may be in part due to a common interaction of each ligand-specific receptor with the gp-130 subunit (Kishimoto et al. 1995). Osteoblastic production of IL-11 is stimulated by many of the same factors that elicit IL-6 including IL-1, TNF-α, PTH and $1,25(OH)_2D_3$ (Romas et al. 1996). In addition to paracrine regulation, IL-11 production from osteoblasts is regulated under autocrine control analogous to autocrine regulation of IL-6 (Heymann et al. 2000). In addition to stimulating RANKL from osteoblasts, IL-11 also has a direct effect upon osteoclasts, resulting in osteoclast proliferation and prevention of apoptosis (Yasuda et al. 1998). Girasole and colleagues suggested that whilst IL-6 subserves osteoclastogenesis induced by estrogen deficiency, in the estrogen replete state IL-11 may play a central role in osteoclast signalling (Girasole et al. 1994). The subsequent discovery of the RANKL/RANK/OPG axis makes this suggestion somewhat less likely.

Thus physiology of bone is highly complex. It is not surprising therefore that analysis of the genetics of osteoporosis should present similar levels of complexity.

1.7 Genetic Studies in Osteoporosis: Association studies

1.7.1 Collagen type I – alpha I chain (COL1A1)

There is convincing evidence of association between a polymorphism in an Sp1 binding site of COL1A1 and BMD. Grant and colleagues demonstrated that the 's' allele of a binding site for the transcription regulator SpI in the first intron of COL1A1 (base +2046, GenBank accession number J03559) was associated with low BMD at the spine, adjusted for height and weight and other confounding variables (Grant et al. 1996). A similar but not significant trend was evident at the femoral neck. The 's' allele was also over-represented in fracture patients compared with controls.

Subsequently several large studies have supported this finding, with association of the 's' allele with both BMD and fracture (Keen et al. 1999; Uitterlinden et al. 1998) in both men and women (Langdahl et al. 1998). Linkage of this gene with BMD has been also demonstrated in twins (Brown et al. 2001). However negative association studies have also been published (Garnero et al. 1998; Heegaard et al. 2000; Hustmyer et al. 1999; Liden et al. 1998; Sowers et al. 1999).

In vitro studies published in abstract form suggested that the 's' allele resulted in greater affinity for Sp1 binding, with as much as three-fold increase in transcription of the 's'

allele compared with the 'S' allele. This could potentially result in an imbalance of α1 chains relative to the α2 chain, affecting bone strength by promoting α1(I) homotrimer formation (Dean et al. 1998). However to date there have been no published articles to confirm the mechanism of the association with osteoporosis.

Further discussion of the genetics of COL1A1 and BMD is presented in Chapter 6.

1.7.2 Vitamin D Receptor (VDR)

There remains considerable controversy about the role of alleles of the vitamin D receptor (VDR) in control of bone density (Eisman 1995; Peacock 1995). Early studies by Morrison and colleagues suggested that VDR polymorphisms were major determinants of serum osteocalcin (Morrison et al. 1992) and bone density (Morrison et al. 1994). A partial retraction of these findings has subsequently been published, mainly due to incorrect allocation of twin heterozygosity (Morrison et al. 1997).

Three common restriction fragment length polymorphic (RFLP) sites recognised by restriction enzymes *BsmI, ApaI and TaqI* have been examined in association studies of VDR, with 'B' or 'b', 'A' or 'a', and 'T' or 't' representing the absence or presence of the RFLP site respectively. *BsmI* and *ApaI* both recognise a noncoding polymorphism located intronically, between exon 7 and the 3' untranslated region. A further RFLP site (C/T) recognised by *TaqI* in exon 9 is a silent polymorphism (both ATT and ATC coding for isoleucine). Linkage disequilibrium between the *BsmI* and *TaqI* polymorphisms of VDR is extremely tight, with >98% concordance between 'b' and 'T' and conversely between 'B' and 't' genotypes (Uitterlinden et al. 1996).

The initial publication by Morrison and colleagues reported both linkage and association between VDR and BMD, with the 'B' allele associated with lower BMD (Morrison et al. 1994). Of note, however, IBD statistics were used to determine linkage when in fact genotypes were only known IBS (no parental information was used). Multitudes of association studies either confirming or contradicting the initial observations have now been published, many of which lacking adequate power to address the question. A large population based survey found no association between any VDR RFLP genotype and BMD (Uitterlinden et al. 1996). A large case-control study of fracture patients did not show any association of VDR genotype with fracture risk (Ensrud et al. 1999), although a similar sized study of similar design did find an association of the 'B' allele with osteoporotic fracture ($p=0.06$) (Langdahl et al. 2000). Two subsequent twin studies had conflicting results (Hustmyer et al. 1994; Spector et al. 1995). One candidate gene linkage study in a small number of families did not show evidence of linkage of BMD and this site (Spotila et al. 1996). A Meta-analysis of 16 studies concluded that VDR polymorphisms had a small effect upon BMD at both hip and lumbar spine. At the hip, the 'at risk' genotype BB resulted in –0.18 SD difference in BMD at the hip and –0.19 SD difference at the spine. There was a trend ($p=0.06$) for VDR polymorphism effects to be greatest in younger age groups, diminishing with age. However, exclusion of the initial publication (Morrison et al. 1994) (not unreasonable given its subsequent partial retraction) resulted in no significant effect of VDR polymorphisms on BMD at hip, spine or distal radius (Cooper et al. 1996). Additionally, heterogeneity of study design significantly influenced spine and hip data ($p<0.0001$ and $p=0.0006$ respectively),

indicating that discrepancies between the types of study could be contributing to their conflicting outcomes.

Several studies have found that the 'BB' or 'tt' genotypes are associated with more rapid bone loss longitudinally (Brown et al. 2001; Ferrari et al. 1995; Gough et al. 1998; Kikuchi et al. 1999; Krall et al. 1995; Yamagata et al. 1994; Zmuda et al. 1997), although even this finding has not been universal (Berg et al. 1996; Garnero et al. 1996; Garnero et al. 1995; Jorgensen et al. 1996; Keen et al. 1995). This may relate to small sample sizes with lack of power to detect a true association, differences in dietary calcium intake (high intake potentially obscuring moderate genetic effects – see below), and the menopausal status of subjects studied (rapid perimenopausal bone loss obscuring non-estrogen related genetic effects).

VDR polymorphisms may affect intestinal calcium absorption (Dawson-Hughes et al. 1997; Wishart et al. 1997), although this finding has not been consistently reproduced (Francis et al. 1997; Kinyamu et al. 1997), and no differences in intestinal VDR expression have yet been demonstrated with different genotypes (Barger Lux et al. 1995). The effect of dietary calcium on bone density in relationship to VDR genotypes has also yielded conflicting results. Brown and colleagues showed that a difference in rates of bone loss was evident between VDR genotypes only in the lowest and middle tertiles of calcium intake, with 'Tt' and 'tt' genotypes having the highest rates of bone loss (Brown et al. 2001). In one study, calcium supplementation had no effect in 'bb' homozygotes but an effect was noted in heterozygotes and a non-significant change in 'BB'

homozygotes (Ferrari et al. 1998), whereas in another study, 'bb' homozygotes on high calcium diets gained bone density compared with other genotypes (Kiel et al. 1997).

Recently, there have been reports of a coding polymorphism (T/C) in exon 2 of the VDR gene resulting in alternative translational start sites and thus different isoforms of the VDR protein (Gross et al. 1996). Women with the longer VDR ('ff' genotype) have been reported to have low bone density in some studies (Arai et al. 1997; Choi et al. 2000; Gross et al. 1996; Harris et al. 1997) but not others (Cheng et al. 1999; Eccleshall et al. 1998; Ferrari et al. 1998; Langdahl et al. 2000; Zmuda et al. 1999). As with the plethora of association studies of the *Bsm1*, *Apa1* and *Taq1* polymorphisms, some of these negative results may be due to small sample sizes with inadequate power to exclude an effect. There is also contradictory data from molecular biological studies into the effects of this polymorphism. One study showed no difference in either the binding of VDR ligands or transactivation of VDR target genes with different genotypes (Gross et al. 1998), and another found less vitamin D-dependent transcriptional activation in the 'ff' compared with 'FF' genotypes (Arai et al. 1997). A study in children found an association between higher calcium absorption and greater BMD with the 'FF' genotype (Ames et al. 1999).

Uitterlinden and colleagues recently reported that although there was no association between VDR and BMD, there was an association between the 'baT' haplotype and fracture risk independently of BMD. The odds ratios for fracture were 1.8 for heterozygous carriers and 2.6 for homozygous carriers, with similar effect at both vertebral and nonvertebral sites. Further, there was evidence of interaction with COL1A1

genotype, such that carriage of both the 'T' allele of COL1A1 (corresponding to the 's' allele discussed above) and the 'baT' allele of VDR further increased the risk of fracture independently of BMD (Uitterlinden et al. 2001).

An association has also been reported between BMD and combinations of polymorphisms of the Estrogen Receptor (ER) and VDR (Gennari et al. 1998; Willing et al. 1998) although, yet again, contradictory results have also been reported (Brown et al. 2001). The power of such studies is again problematic, as division into multiple genotype groups reduces the sample size of each.

It therefore remains possible that this gene or linked genes may contribution to the heritability of bone mineral density and/or fracture. However the effect may be evident only in particular environmental or genetic backgrounds.

1.7.3 Estrogen Receptor-alpha (ER-α)

Three SNPs recognised by restriction enzymes *BstUI* (in exon 1), *PvuII* and *XbaI* (both in intron 1) and a dinucleotide microsatellite marker in the promoter region have produced suggestive evidence for a role for ER-α in determining bone density.

Association of the RFLPs and bone density in postmenopausal women was initially reported in Japanese (Kobayashi et al. 1996) and Caucasians (Gennari et al. 1998; Willing et al. 1998). However, other groups have found no association in postmenopausal women (Bagger et al. 2000; Han et al. 1997; Vandevyver et al. 1999) of the same and different ethnic groups. In Thailand, association with the opposite genotype

was seen in males (Ongphiphadhanakul et al. 1998) and in young premenopausal females (Ongphiphadhanakul et al. 1998).

One study of pre-, peri- and postmenopausal women did not find an association overall but found an association with the heterozygous Xx genotype in younger premenopausal women. Further, markers of bone turnover were greater in the perimenopausal Xx genotype compared with the xx genotype. This suggests that the effects of ER-α on BMD may be greatest in younger people (and thus affect peak bone mass), with ablation of its effects with the bone loss of menopause. However, the numbers involved were very small (Mizunuma et al. 1997).

There is strong linkage disequilibrium between the RFLPs identified in the ER-α (Becherini et al. 2000; Langdahl et al. 2000). Different haplotypes have been associated with low bone density in different studies. The 'Px' haplotype was found to have lower bone density in Japanese postmenopausal women (Kobayashi et al. 1996) and in a British cohort of mostly (92.7%) postmenopausal women (Albagha et al. 2001) whereas the 'PX' haplotype was associated with lower bone density in a study of Italian postmenopausal women (Gennari et al. 1998). It should be pointed out that several of these studies use probabilistic rather that actual haplotypes for their statistical analysis (Becherini et al. 2000; Gennari et al. 1998; Kobayashi et al. 1996; Langdahl et al. 2000).

Recently three studies examining the three RFLPs in the 1st exon and intron and a (TA) repeat in the promoter region have demonstrated linkage disequilibrium between all the polymorphisms (Albagha et al. 2001; Becherini et al. 2000; Langdahl et al. 2000). Despite finding no association with the intragenic polymorphisms, two studies reported

an association between the promoter region and BMD, with fewer (TA) repeats associated with lower BMD (Becherini et al. 2000; Langdahl et al. 2000). In contrast, the third study reported an association with the Px haplotype but none with the (TA) repeat polymorphism, with greater number of (TA) repeats in linkage disequilibrium with the 'at risk' Px haplotype (Albagha et al. 2001). The interpretation of such studies is difficult as the level of cut-off of repeats is arbitrary and critically alters results. A further study of the (TA) repeat polymorphism found an association with BMD at LS, although this result was not significant after adjustment for hysterectomy and oopherectomy (Sowers et al. 1999).

Longitudinal studies of ER-α polymorphisms and bone loss demonstrated contradictory results. No association was seen between ER-α and BMD in a group of elderly postmenopausal women (Bagger et al. 2000; Brown et al. 2001), nor in a perimenopausal group (Willing et al. 1998), although an association was found in one young postmenopausal cohort (Salmen et al. 2000).

Two studies have suggested that there is genetic interaction between the VDR and ER-α influencing bone density (Gennari et al. 1998; Willing et al. 1998). No effect of either ER-α or VDR/ ER-α combined on bone density or change in bone density was demonstrated in a study of late postmenopausal women, also suggesting that this effect may be most prominent in younger individuals (Brown et al. 2001).

A study of body size in infants found a similar interaction between ER-α and VDR and an independent association of the *PvuII* ER-α SNP with body growth in infancy, suggesting that this interaction may primarily influence peak bone mass (Suarez et al.

1998). An association of the *PvuII* ER-α SNP with BMI in adults has also been reported (Deng et al. 2000).

An association has been shown between ER-α alleles and QUS (Patel et al. 2000). Further, this study also showed that another gene associated with the intracellular transduction of estrogen signals (estrogen receptor cotranscriptional activator amplified in breast cancer-1) had both an independent association with QUS and also interacted with alleles of ER-α in determining BMD.

1.7.4 Interleukin-6 (IL-6)

A study looking at a minisatellite in the 3' flanking region of IL-6 reported an association with low bone density in homozygotes (genotype 'FF') compared with heterozygotes (genotype 'CF'), although of note the 'CC' homozygotes actually had lower lumbar spine bone density compared with either 'C/F' or 'F/F' (Murray et al. 1996). A similar trend was noted at the femoral neck. A study of Japanese postmenopausal women examining a different microsatellite at this locus found an association with radial bone density (Tsukamoto et al. 1999). A polymorphism in the promoter region of IL-6 has been reported to affect IL-6 transcription and plasma IL-6 levels (Fishman et al. 1998), and an association study of this polymorphism in young men reported association with peak BMD (Lorentzon et al. 2000). Linkage has also been reported between osteopaenia (as a qualitative trait) and IL-6, but not with IL-6R (Ota et al. 1999), although other studies have not confirmed this (Takacs et al. 2000).

1.7.5 Transforming Growth Factor-β (TGF-β)

Polymorphisms of the TGF-β1 gene have been associated with low BMD, fracture and increased bone turnover. Association of an intronic single base deletion affecting the splice junction sequence upstream of exon 5 (713-8delC) with low bone density, fracture and increased bone turnover has been reported in two studies (Bertoldo et al. 2000; Langdahl et al. 1997). Additionally bone-specific alkaline phosphatase, a marker of bone formation, was also associated with this polymorphism. A study of Japanese postmenopausal women did not find the 713-8delC deletion in the study population. However a different polymorphism (T/C transition at nucleotide 29 in the signal sequence region causing a Leu to Pro substitution at AA position 10) was found to be associated with low bone density, fractures, and increased TGF-β1 serum levels with a trend for increased markers of bone resorption (Yamada et al. 1998). Longitudinal studies showed greater bone loss in patients with the 'TT' genotype, with significantly greater response to $1,25(OH)_2D_3$ therapy and a trend to greater response to hormone replacement therapy in patients with the 'CC' genotype (Yamada et al. 2000). A study of yet another TGF-β1 polymorphism, a T/C polymorphism in intron 5, also reported association with BMD (Keen et al. 2001). Serum levels of TGF-β1 have been reported to have heritability of 0.54 (Grainger et al. 1999).

1.7.6 Interleukin-1

The Interleukin-1 family includes IL-1α, IL-1β and the IL-1 receptor antagonist (IL-1RA). Two association studies of IL-1RA have been performed in osteoporotic patients (Keen et al. 1998; Langdahl et al. 2000), both finding a weakly significant association.

Keen and colleagues found an association of early postmenopausal bone loss at the spine but not the femoral neck with the 'A1 A1/A3' genotypes (Keen et al. 1998). Langdahl and colleagues found an association of the same genotype with both osteoporotic fracture and low BMD at the spine. Although there was no association with any markers of bone turnover, they noted a non-significant trend of increasing difference in LS BMD between genotypes with increasing age (Langdahl et al. 2000). Of note, though, the heterozygote group had the highest BMD compared with the homozygotes of both the putative 'at-risk' and the 'protective' alleles, which is biologically improbable.

1.7.7 TNF-α/MHC

A single linkage study in Japanese women, analysing BMD as a qualitative trait, reported linkage of TNF-α to BMD, using an intragenic microsatellite marker, although the p-value threshold used to report linkage was unusually high (Ota et al. 2000). However, as linkage disequilibrium across the MHC is extremely high, this result at best suggests linkage of MHC rather than of TNF-α *per se*. A previous small association study of HLA also in Japanese women had also reported association with a particular MHC haplotype (Tsuji et al. 1998).

1.7.8 IGF-1

Association was reported with an intragenic CA-repeat and BMD in a small study focussing on males with osteoporosis (Rosen et al. 1998). Subsequent larger linkage and association studies have not supported this result (Takacs et al. 1999).

1.7.9 Parathyroid Hormone (PTH)

Two association studies of an intronic *BstB1* RFLP site in the PTH gene and BMD have been performed, with contradictory findings (Hosoi et al. 1999; Johnson et al. 1995). A further study suggested an effect upon bone size rather than on BMD (Gong et al. 1999).

1.7.10 Calcitonin Receptor

Two studies of different polymorphisms of the calcitonin receptor have reported association with low BMD and fracture (Masi et al. 1998; Taboulet et al. 1998). However, in both of these, BMD was reported to be highest in the heterozygote groups (with low significance levels), which is not consistent with a biological role of these polymorphisms in determining BMD.

1.7.11 Osteocalcin

Serum osteocalcin levels have been reported to be a heritable trait. Greater correlation was observed in serum osteocalcin levels between monozygotic twins than in dizygotic twins, both pre- and postmenopausally (Kelly et al. 1991). The allocation of zygosity for some twin pairs in this study has since been demonstrated to be incorrect (Haughton et al. 1998).

Allelic variation of the VDR is correlated with serum osteocalcin levels, suggesting that population variation in VDR may underlie variance in BMD through its effects upon osteocalcin production and, by inference, bone formation (Morrison et al. 1992).

In a modest sized association study involving 261 women, no association was demonstrated between a dinucleotide CA repeat tightly linked to the osteocalcin gene, and serum osteocalcin levels either at baseline or with change over 3 years. Additionally, there was no association between osteocalcin genotype and BMD at baseline or with its change over 3 years (Willing et al. 1998). However, this negative study did not have the power to address the question due to the number of genotypes examined. A further study by this group of a C/T polymorphism in the promoter region of osteocalcin was also negative (Sowers et al. 1999). An association study in Japanese women of a C/T polymorphism in the osteocalcin promoter region did not show a significant difference in BMD between genotypes (Dohi et al. 1998).

1.7.12 Osteopontin

An association study of an intragenic dinucleotide repeat in the osteopontin gene and BMD showed a significant association with baseline BMD at femoral neck but not at other sites. The bulk of the contribution to the chi-squared statistic was due to a single heterozygous genotype without any clear allele-dose effect. There was no association between genotype and change in BMD over 3 years (Willing et al. 1998).

1.7.13 Others

Other candidate genes have been reported to have associations with bone density, including the apolipoprotein E gene (Salamone et al. 2000; Shiraki et al. 1997) and the calcium sensing receptor (Tsukamoto et al. 2000). As with many of the above genes, the

level of significance obtained in these studies has not been great, and confirmation studies will be required to determine their general relevance.

1.8 Genetic Studies in Osteoporosis: Linkage Studies

Very few large linkage studies have been performed in osteoporosis. A candidate gene study in a small number of ethnically heterogeneous families did not demonstrate any linkage with several candidate genes (VDR, COL1A1 or COL1A2), although the study did not have adequate power to exclude significant effects (Spotila et al. 1996). Subsequently a whole genome scan was performed in this sample of families, with suggestive evidence of linkage of BMD to loci on chromosomes 1p, 2p and 4q, with the highest individual marker lying on chromosome 11q (LOD 2.08 at marker CD3D) (Devoto et al. 1998). A genome-wide scan in 96 Chinese families with 153 sib-pairs showed linkage of forearm bone mineral density with several areas of chromosome 2 and a more localised area on chromosome 13 (Niu et al. 1999).

A genome-wide scan in a family kindred (22 members) with autosomal dominant high bone density showed linkage to chromosome 11q12-13 with maximal LOD score (MLS) of 5.74 at marker d11s987. As individuals with high bone density were identifiable by young adulthood, this locus was therefore thought to be involved in determination of peak bone mass (Johnson et al. 1997). A linkage study of 374 Caucasian and African-American sisterships initially examining only 56 cM of chromosome 11q12-13 showed linkage of this region to femoral neck bone density (LOD score 3.5) and to a lesser extent

lumbar spine BMD (LOD score 1.63 in Caucasian sisters only) (Koller et al. 1998). Subsequently a full genome scan was performed in an expanded cohort, with significant evidence of linkage of chromosome 1q21-23 with lumbar spine BMD. Suggestive evidence of linkage was seen at chromosomes 5q33-35 and 11q12-13 with femoral neck BMD, and at 6p11-12 with lumbar spine BMD. The area of 11q12-13 had a lower LOD score with their expanded population than in their earlier work (Koller et al. 2000).

As indicated above, three studies have shown linkage of bone mineral density to an area on chromosome 11q. Two monogenic diseases associated with abnormal bone density (autosomal recessive osteopetrosis (Heaney et al. 1998) and osteoporosis-pseudoglioma syndrome (Gong et al. 1996)) have been mapped to the same region. It seems likely that this region will contain a gene or genes relevant to the control of bone density in the general population.

Recently two small candidate gene linkage studies have reported linkage of BMD to IL-6 (Ota et al. 1999) and and to TNF-α (Ota et al. 2000).

So in summary, despite many association studies of BMD and various genes, there have been few candidate or whole genome linkage studies in osteoporosis, with none performed in a homogeneous population looking at BMD of lumbar spine and femoral neck. The genes underlying population variance in BMD remain unknown. This thesis presents work undertaken in order to determine such genes.

Chapter 2: Materials and Methods

2.1 Genomic DNA Extraction

DNA was extracted from peripheral blood mononuclear cells by a modification of the guanidine hydrochloride method (Jeanpierre 1987).

Peripheral venous blood was collected in EDTA-containing tubes (Greiner, UK). Following freeze-thawing, 30mL of blood was mixed with approximately 30mL of erythrocyte lysis buffer (1% Triton-X 100, 320 mM sucrose, 1 mM Tris(hydroxymethyl)methylamine pH 8, 5mM magnesium hydrochloride). This was vortexed then centrifuged at 2500rpm for 15 minutes. The supernatant was poured off and the pellet resuspended in approximately 20mL of lysis buffer, vortexed and centrifuged again. This process was repeated until there was little visible haemoglobin remaining.

The pellet was resuspended in 3.5mL 6M guanidine hydrochloride and 250μL 7.5M ammonium acetate and vortexed. Proteinase K 50μL (10mg/mL in 1% lauryl sulfate, 2mM sodium edetate pH 8.0) and 250μL 10% SDS were added to the solution. The mix was then incubated either at 37° overnight or at 60° for an hour. The mixture was then cooled to room temperature. 2mL of chloroform was added, the tube vortexed and then centrifuged at 2000 rpm for 3 minutes. The aqueous supernatant was collected by pipette and added into a fresh tube of 10mL of cold 100% ethanol (–20°C). This tube was then agitated to precipitate the DNA. The tube was centrifuged for 15 minutes at 3000rpm. The supernatent was drained, the DNA pellet washed twice with 70% ethanol and

transferred to a 1.7mL Eppendorf tube, and the pellet left to dry. The DNA was then resuspended in 1M TE8.

2.2 Spectrophotometric Quantification of DNA.

The concentration and purity of DNA samples were assessed by UV light absorption. 5μL from each DNA sample was diluted 1 in 20 in SDW and then aliquoted into a cuvette. The optical density was assessed at light wavelength 260nm and 280nm using a spectrophotometer (Beckton-Dickinson). The concentration of DNA is given by the formula:

$$\text{Concentration (ng/}\mu\text{L)} = OD_{260} \times \text{dilution} \times 50.$$

The purity was assessed using the ratio OD_{260}/OD_{280}. The absorption at 280nm is mainly due to protein contaminants. Pure DNA has a ratio ≥1.5.

2.3 Microsatellite Amplification and Detection

2.3.1 The use of Microsatellite Markers and Polymerase Chain Reaction For Linkage

The power of linkage analysis depends critically upon the informativity of the markers used (Risch 1990). The probability that a marker will be informative for linkage analysis depends on the number of alleles of the locus and their frequency. Parents homozygous for a marker will not be informative for linkage as IBD status in the offspring cannot be determined (unless multipoint analysis is performed). Similarly, parents who are

identical heterozygotes will also not be informative for linkage when the offspring are also heterozygotes, as again IBD status cannot be determined. The proportion of meioses that will be informative at a marker is given by the polymorphism information content (PIC) of that marker. This is equal to one minus [(the proportion of meioses involving homozygotic parents) and (the proportion of meioses occurring from identical heterozygotic parents resulting in heterozygotic offspring)].

$$PIC = 1 - (\sum_{i=1}^{n} p_i^2) - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 p_i^2 p_j^2$$

Where p_i is the frequency of allele i

n is the number of alleles at the marker locus

$2p_ip_j$ is the proportion of heterozygotes (equation 1)

$4p_i^2p_j^2$ is the proportion of parents who are identical heterozygotes (equation 2)

$2p_i^2p_j^2$ is the proportion of meioses involving identical heterozygous parents that result in heterozygotic offspring at a particular marker (equation 3).

Note that equation 2 is the square of equation 1, and equation 3 is half equation 2.

Microsatellite markers are short sequences of tandem nucleotide repeats, the bulk of which are dinucleotide $(CA)_n$ repeats. Microsatellite markers are spread throughout the genome and are common, with (CA) repeats accounting for 0.5% of the total genome (Strachan et al. 1996). Microsatellites are highly polymorphic i.e. the number of repeats is highly variable between different alleles present in the population, making it unlikely that an individual will be homozygous for a particular marker. Microsatellites are also

easily typed by semiautomated means. Of note, $(CA)_n$ repeats are prone to replication slippage during PCR, so that stutter bands may be evident on both agarose and genotyping gels. This combination of characteristics means that microsatellite markers are very useful for linkage analysis. However, they are likely to be superseded by single nucleotide polymorphism technology (see Chapter 1: Section 1.5.3).

Microsatellite markers are amplified by polymerase chain reaction (PCR). PCR is a means of cloning multiple (in the order of 2^{32}) copies of small segments of DNA. In brief, oligonucleotide probes (known as primers) anneal to the DNA flanking the microsatellite variable region and promote replication of the intervening sequence by providing starter templates for DNA polymerase.

2.3.2 PCR Protocols

PCR of microsatellite markers was undertaken in 96 well V-bottom microtitre plates (Costar, High Wycombe, UK). The reaction mix consisted of 50ng DNA, 0.4μL of 5μM primer solution (containing both forward and reverse primers), 0.25μL of 2mM dNTPs, 0.5-3.5mM $MgCl_2$, 1μL 10xNH_4 buffer, 0.2 units of DNA polymerase and SDW to 10μL volume. Unless otherwise specified, the DNA polymerase used was Bioline Taq (Bioline UK Ltd, London, UK). MJ thermal cyclers (MJ Research, Watertown, MA, USA) were used for PCR, with standard cycling conditions of DNA denaturation (94°C 1 minute), annealing of probes (54-61°C for 1 minute) and extension (72°C for 45 seconds). Annealing temperature, magnesium concentration and numbers of cycles for each microsatellite were individually optimised.

For genotyping using ABI373 machines, primers were synthesised with a fluorescent tag (FAM, ROX, HEX, TET) from Sigma-Genosys Ltd., Pampisford, Cambridgeshire, UK; Gibco/Life Technologies™, Paisley, UK or were selected from the Medical Research Council (UK) set of primers (Reed et al. 1994).

2.3.3 Use of de-aza deoxyguanosine trisphosphate

DNA with high (G+C) content has substantial intra-strand secondary structure that during normal electrophoresis is not fully denatured. This results in anomalous migration with adjacent bands of DNA becoming compressed. Compression is entirely dependent on secondary structure. To diminish secondary structure, nucleotide analogues can be used: either dITP (deoxyinosine trisphosphate) or 7-deaza-dGTP. These pair weakly with conventional bases whilst being good substrates for DNA polymerases: thus they are well incorporated and diminish secondary structure resulting in fewer compressions and facilitating work with (G+C) rich DNA (Sambrook et al. 1989).

2.4 Agarose Gel Electrophoresis.

3% agarose gels were prepared by dissolving 3g agarose (Sigma, Poole, UK) in 100mL 1xTBE (0.09M Tris-borate, 0.002M EDTA) and boiling in a microwave oven. Ethidium bromide (8μL of 10mg/mL solution) was added to the cooled agarose solution which was then poured into the gel mould.

Alternatively, agarose gels were poured without adding ethidium bromide, but subsequent to electrophoresis the gels were soaked in a solution of ethidium bromide (1g/L in TBE) for 30-60 minutes.

5μL of loading buffer (30% glycerol in sterile distilled water with equal mass of bromophenol blue and xylene cyanole (approximately 0.1g of each for 100mL solution)) was mixed with an equal volume of PCR product prior to loading. DNA marker VIII (Boehringer Mannheim, Lewes, UK) was used as a size standard.

Gels were electrophoresed in 1xTBE at 150-200V for at least 15 minutes. Nucleic acid was then visualised by UV light transillumination, as ethidium bromide intercalates with DNA and fluoresces under UV light.

2.5 Polyacrylamide Gel Electrophoresis for Microsatellite Markers

Separation of microsatellite markers was performed by electrophoresis using ABI 373 semiautomated sequencers (Applied Biosystems, Warrington, UK) using 6% denaturing polyacrylamide gels.

The PCR products were diluted with water according to the amount of PCR product present, and pooled into primer sets. These were arranged such that no set contained two primers of the same length with the same colour fluorescent tag.

For each sample, 2.4μL of loading buffer (8.3mg/mL blue dextran, 5mM EDTA, 86% formamide) was mixed with 0.6μL GS Tamra 500 size standard (see below) and 3-5μL

of pooled PCR product. The mix was denatured at 95° for 3 minutes then rapidly cooled on ice. 2-3μL of the mix was loaded per well. Gel electrophoresis was performed at approximately 900V for approximately 4 hours (according to the size of the largest PCR product).

2.6 Size standards

2.6.1 Size standards for agarose gels

Marker VIII (Boehringer Mannheim, Lewes, UK) is a mixture of digestion products of the plasmid pUCBM21 by the restriction enzymes *Hpa II* and separate digestion with *Dra I* and *Hind III*. 100μL Marker VIII was mixed with 1.55mL TE8 and 1.65mL loading buffer to make a size standard for running with agarose gels. The bands produced are shown in the table below.

2.6.2 Size standards for genotyping gels

GS Tamra 500 (Perkin Elmer, Boston, Massachussetts, USA) is made by *Pst I* digestion of plasmid DNA followed by ligation of a TAMRA-labelled 22-mer to the cut ends. Subsequent digestion with *Bst UI* produces DNA fragments, each containing a single TAMRA dye. 0.6μL of GS Tamra 500 was mixed with 2.4μL of loading buffer for each sample loaded. The bands produced are shown in the table below.

Table 2.6: Size standard bands

Band	Marker VIII	GS Tamra 500
1	1114	500
2	900	490
3	692	450
4	501	400
5	489	350
6	404	340
7	320	300
8	242	250
9	190	200
10	147	160
11	124	150
12	110	139
13	37	100
14	37	75
15	34	50
16	26	35
17	19	

2.7 Semiautomated Genotyping of Microsatellites.

Products were sized using the programme GeneScan™ Versions 2.0.2 and 2.1 (Applied Biosystems, Warrington, UK) and genotypes semiautomatically assigned using the programme Genotyper™ Version 1.1 (Applied Biosystems, Warrington, UK). All genotypes were manually checked. The programme GAS (A. Young, unpublished) was used to convert the size data into discrete allele numbers. Mendelian inheritance was

checked both manually, using the programme Genotyper^{TM}, and automatically, using GAS (A.Young) and Pedcheck (O'Connell et al. 1997).

Allele frequencies were calculated from the data using the programme Downfreq (J.Terwilliger, unpublished).

Allele sharing and linkage were assessed using the programmes Mapmaker/Sibs (Kruglyak et al. 1995), Analysis of Complex Traits ('ACT') (Amos et al. 1996), Sequential Oligogenic Linkage Analysis Routines (SOLAR) (Almasy et al. 1998), and Quantitative Transmission Disequilibrium Testing (QTDT) (Abecasis et al. 2000). These programmes are discussed in more detail in Chapters 1,4,6, and 7.

2.8 Denaturing High Performance Liquid Chromatography (DHPLC)

2.8.1 DHPLC Rationale

Heteroduplex analysis is a frequently used mutation screening method. Heteroduplexes arise from the annealing together of a wild type DNA strand with its complementary mutant strand, resulting in one or more mismatched bases. Examples of heteroduplex analysis detection systems include heteroduplex mobility in polyacrylamide gels (utilising the different electrophoretic mobility of heteroduplexes and homoduplexes in polyacrylamide gels), chemical or enzymatic cleavage of mismatches (CCM or ECM) present in heteroduplexes, and denaturing gradient gel electrophoresis (differing migration of the different DNA duplexes through a gel with increasing gradient of either

chemical or temperature denaturant; migration ceases when the DNA denatures). Heteroduplex based analysis systems will not pick up homozygous mutations, unless the sample has been mixed with wild-type DNA. In contrast, single strand conformation polymorphism (SSCP) analysis, another frequently used mutation detection method, relies on differences in folding patterns and secondary structure of single stranded DNA. SSCP does not rely upon heteroduplex formation although again homozygous mutations would not be detected unless a control wild type was run for comparison. However, commonly used methods of mutation detection (such as SSCP and heteroduplex mobility in polyacrylamide gels) lack sensitivity (Sheffield et al. 1993), whilst more sensitive methods (such as denaturing gradient gel electrophoresis and CCM) are expensive and labour intensive (O'Donovan et al. 1998).

Denaturing high performance liquid chromatography (DHPLC) is a rapid and sensitive means of mutation detection capable of detecting single base pair changes. A sample from an individual who is heterozygous for a single nucleotide polymorphism or mutation will have 1:1 ratio of wild type and mutant DNA. After amplification by PCR (see below), the DNA samples are denatured by heating to 95°C followed by slow cooling to promote the formation of heteroduplexes. Thus after hybridisation four species of DNA will be present from a heterozygous individual – 2 homoduplexes and 2 heteroduplexes. The PCR product is then analysed using ion-pair reverse phase liquid chromatography. This is undertaken at a temperature sufficient to partially denature the sample. Under non-denaturing conditions the four species of DNA will all have the same retention time on the column or matrix. As the temperature increases gradually, the heteroduplex DNA fragments denature in the region around the mismatched base pair(s).

This denaturation results in a reduction of the double-stranded portion of the PCR product. Heteroduplex fragments containing the mismatches will have a greater proportion of single-stranded DNA and therefore will elute off the column before homoduplex fragments. Retention time of each homoduplex fragment and of each heteroduplex fragment will differ due to the differing base constitution of each species: an A-T homoduplex will denature and elute before a C-G homoduplex (due to the additional hydrogen bond existing between the C-G base pair). Therefore the differing species will be resolvable into two sets of two fragments representing both homoduplexes and both heteroduplexes. At sufficiently high temperatures all species will be denatured (Kuklin et al. 1997).

Samples are amplified using touchdown PCR using both a 5'-3' DNA polymerase and additional 3'-5' proof-reading exonuclease. Touchdown PCR results in the first strand synthesis occurring at the highest possible temperature and thus having the highest specificity; subsequent amplification occurs from this template. This results in fewer species of DNA from non-specific primer amplification ('mis-primed products'). The proof-reading enzyme results in fewer PCR-induced errors. The fidelity of PCR is critical, as detection of mis-primed products or error induced by PCR will be confused with the detection of true heterozygotes.

DHPLC is performed at a temperature sufficient to partially denature the DNA complexes. The clearest definition between different fragments occurs with approximately 10% denaturation, i.e. 80-90% helical fraction or 'double strandedness'. This is fragment-specific, with higher temperatures needed for denaturation of (G+C) rich

areas compared with (A+T) rich fragments. The appropriate denaturation conditions can be calculated using the theoretical T_m from sequence data and various software packages (e.g.WAVEMaker™ 3.3, Transgenomic Inc., San Jose, California, USA). Alternatively, the appropriate temperature for 90% denaturation can be determined by experiment, running incremental temperature curves. These plot retention time of wild type DNA against temperature, starting at approximately 50°C (DNA fully double-stranded) and increasing temperature until the DNA is fully denatured. Once the appropriate temperature is determined, the quality of the PCR product can be tested using control wild type DNA. PCR fidelity can be assessed from the shape of the curve of retention time, looking for evidence suggesting the presence of more than one DNA fragment, in which case the PCR reaction must be re-optimised. Longer sections of DNA may have more than one domain of melting (i.e. 10% denaturation of each domain may occur at different temperatures according to the different base pair constitution) and thus it may be necessary to analyse samples at more than one temperature. On average, two temperatures are needed.

For mutation detection, 50μL of PCR product is analysed by the WAVE™ DNA Fragment Analysis System (Transgenomic Inc., San Jose, California, USA). The WAVE machine is an automated system allowing plates of up to 96 samples to be analysed sequentially and rapidly (6.8 minutes/sample). This system is capable of detecting single base changes in fragments as large as 1.5 kB, although the optimum length is between 150 and 450 base pairs (Kuklin et al. 1997). Larger products are also more likely to have more than one domain of melting and, as indicated above, may require several analyses at differing temperatures. The samples anneal to a high resolution matrix consisting of

polystyrene-divinylbenzene copolymers (DNASep^R, Transgenomic Inc., San Jose, California, USA). The buffers used for mutation detection consist of a) 0.1M triethylammonium acetate (TEAA) and b) 0.1M TEAA with 25% acetonitrile. DNA binds to the column through the bridging molecule TEAA: the alkyl group binds to the column whilst the ammonium ion binds to the phosphate ions of the DNA molecule. The former interaction is disrupted by an increasing gradient of acetonitrile concentration, achieved by a 2% increment in buffer B per minute. Thus at the predetermined temperature for partial denaturation, the four different DNA species will elute at slightly different concentrations of acetonitrile and will be detected by UV screening of the buffer diluent. Typically heterozygotes will elute at concentrations of buffer B 1.5 to 2% lower than is required for elution of the homozygotes (Taylor et al. 1998).

Populations containing a high number of homozygous mutants can be screened by addition of known wild-type DNA to the unknown sample. Normally homozygous mutants will not be detected, as the sample will elute as a single entity, indistinguishable from a homozygous wild type sample. Addition of control wild type DNA will allow for the formation of heteroduplexes with the mutant homozygous DNA; the mutation will thus be detected. The addition of control wild type DNA to heterozygous samples is not detrimental to their detection despite disrupting the usual 1:1 ratio between wild type and mutant DNA strands (Escary et al. 1999). Indeed mutations may be detected with a wild type to mutant DNA ratio of 50:1 (Kuklin et al. 1997). If DHPLC is run as a single step with the addition of wild type DNA to the samples, homozygous mutants and heterozygous samples would be indistinguishable. To distinguish clearly between

homozygous wild type, heterozygous and homozygous mutant samples requires a two-step procedure.

DHPLC is a highly sensitive and specific method for detecting mutations ((O'Donovan et al. 1998), (Liu et al. 1998). It is more sensitive than SSCP or gel-based heteroduplex analysis (Jones et al. 1999), although SSCP has the advantage of reliable identification of different sequence variants (Dobson-Stone et al. 2000).

2.8.2 PCR Protocol for DHPLC

PCR for DHPLC was performed with oil-free conditions in 0.2mL skirted 96 tube plates (ABgene®, Epsom, Surrey) with microcap covers (ABgene®, Epsom, Surrey) on MJ Thermal Cyclers (MJ Research, Watertown, MA, USA).

For each 50μL sample, 50ng DNA was used as template with 2μLx 5mM primers (both forward and reverse), 1.25μL x 2mM dNTPs, 5μL of 1 x Expand^{TM} High Fidelity PCR buffer (Boehringer Mannheim, Lewes, UK), 1.225 units Expand^{TM} High Fidelity PCR enzyme (Boehringer Mannheim, Lewes, UK), $MgCl_2$ at 0.5-3.5mM and sterile distilled MilliQ water to achieve final volume. When used, dimethyl sulfoxide (DMSO) was included at 2% concentration.

The PCR protocol consisted of 94°C for 2 minutes, followed by touchdown protocol ([94°C for 1 minute], [(7.5°C + final annealing temperature, minus 0.5°C per cycle) for 1 minute], [72°C for 45 seconds]) repeated 14 times, then 25 cycles of ([94°C for 1 minute], [final annealing temperature for 1 minute], [72°C for 45 seconds]). PCR was finished with 10 minutes at 72°C.

To promote heteroduplex formation, samples were then denatured by heating to 95°C for 4 minutes, then cooled over 45 minutes to a final temperature of 25°C.

Magnesium concentration and annealing temperature were optimised for each primer individually.

2.9 Sequencing

2.9.1 Sequencing Rationale

Sequencing determines the exact base composition of DNA and is a means of exact identification of polymorphisms. Most sequencing now involves enzymatic amplification of a single strand DNA template. The nucleotides used in synthesis include 2',3'-dideoxynucleotides (ddNTPs) that lack hydroxyl groups at both the 3' carbon position and the usual 2' position. Thus, although ddNTPs can be conventionally incorporated into the synthesising chain of DNA through their 5^{th} carbon position, they cannot form a phosphodiester 3'-5' bond to extend the DNA chain to the next nucleotide, resulting in chain termination. By using a mix of ddNTPs and dNTPs with very low ddNTP concentration, the incorporation of the ddNTP will occur randomly and result in a whole series of DNA fragments of different sizes. The fragments will have a common 5' end (the original priming template) but variable 3' end according to the point of termination. These fragments can then be separated using a denaturing polyacrylamide gel. Detection of the different sized fragments involves either labelling the primer or the ddNTPs.

'Dye terminator' sequencing using fluorophore-labelled ddNTPs means that only one synthesis reaction is needed. 'Dye-primer' sequencing using fluorophore-labelled primers can also be performed: four separate reactions (one for each ddNTP), each with a different labelled primer, would be needed but the collection of DNA fragments could be pooled and run in a single lane. During electrophoresis, the DNA fragments pass a laser beam that excites the dyes and causes fluorescence at a different wavelength for each dye. This information is detected and stored by the sequencing machine for later analysis.

2.9.2 Sequencing

For the sequencing in this study the dyes used were dichloro-rhodamine dye terminator ddNTPs (DR110/Fam, DR6G/Fam, DTamra/Fam and DRox/Fam), supplied as 'BigDye' kit (Applied Biosystems, Warrington, UK). The sequencing reaction mix consisted of 8.8μL post PCR DNA product, 4μL BigDye kit, 4μL half strength BigDye and 3.2μL of either forwards or reverse primer using 1mM stock solution. The control was 2μL of PGem DNA with 4μL of 0.8mM primer, 4μL BigDye kit, 4μL half strength BigDye and sterile distilled water to 20μL.

The cycling conditions were ([96°C for 10 seconds], [50°C for 5 seconds], [60° for 4 minutes]) for 25 cycles using MJ thermal cyclers (MJ Research, Watertown, MA, USA).

After PCR the product was added to 100μL 100% ethanol on ice for 10 minutes before centrifuging at 13000 for 10 minutes. The supernatent was removed and the pellet was gently resuspended in 150μL of 70% ethanol. After brief centrifuging the ethanol was

completely removed and the pellet left to air-dry for 5-10 minutes. If not immediately used the samples were stored at –20°C.

Just prior to loading the samples, 3μL of loading buffer (8.3mg/mL blue dextran, 5mM EDTA, 86% formamide) was added to each sample. The sample was denatured at 95°C for 2 minutes then cooled rapidly on ice and loaded on the sequencing gels. Sequencing was carried out using ABI 377 automated sequencers (Applied Biosystems, Warrington, UK), and 40% acrylamide gels.

Sequencing was analysed using the programmes Factura™ 2.0.1 and Sequence Navigator™ (both Perkin Elmer, Applied Biosystems, Warrington, UK).

2.10 QIAquick PCR Purification

Where samples required purification prior to sequencing, the QiaQuick PCR Purification Kit was used following the given protocol (Qiagen, Crawley, West Sussex).

2.11 Restriction Fragment Length Polymorphism (RFLP) Typing

Restriction endonucleases recognise specific, often palindromic, sections of DNA. In the presence of a restriction enzyme site, the endonuclease will bind to the DNA and cleave it into fragments of specific length, according to the sites of cleavage. A single base pair change in the recognition sequence is sufficient to prevent enzyme binding and activity. Thus RFLP typing can be used to detect single nucleotide polymorphisms as a di-allelic system.

The DNA section of interest is amplified by PCR as described above. The PCR product is incubated with the endonuclease (at enzyme-specific temperature and duration). If an RFLP site is present the allele will be cleaved into a characteristic length. The fragments are separated and detected by agarose gel electrophoresis (as described above).

Chapter 3: Genetic Epidemiology of Osteoporosis and Bone Mineral Density

3.1 Introduction

Both low BMD and fracture are heritable traits. One of the greatest risk factor for hip fracture for white women is a maternal history of hip fracture (Cummings et al. 1995). Twin, mother-daughter pair, and family studies have demonstrated that BMD is a highly heritable trait (reviewed in Chapter 1). Heritability of peak bone mass may be the major component of heritability of BMD overall (Gueguen et al. 1995; Jouanny et al. 1995). However, there is some evidence of heritability of bone loss also so that heritability of bone loss may become an increasingly important component of overall BMD heritability with age (Garnero et al. 1996; Hansen et al. 1992; Harris et al. 1998; Kelly et al. 1991; Tokita et al. 1994). Heritability of BMD and fracture may differ at different skeletal sites, either due to effects of different genes upon different sites or to differing gene-environment interaction. Further, there may be both common and gender-specific effects upon BMD, which again may be site-specific. Finally, heritability of BMD assessed by DXA may be different from heritability of QUS (Arden et al. 1996).

This chapter presents genetic epidemiology of a cohort of families recruited for genetic studies of BMD and osteoporosis.

3.2 Proband and Family Recruitment

To be eligible for this study, probands needed to fulfil two criteria:

a) Primary osteoporosis, defined by WHO (t-score ≤ –2.5 at either LS or FN) (Kanis et al. 1994)

b) Low BMD according to an age- and gender-matched cohort. This was arbitrarily set at a z-score ≤ –2.0 at either FN or LS.

Probands were recruited from several sources. Both male and female probands were identified from the osteoporosis clinics of the Department of Endocrinology and Metabolism, Nuffield Orthopaedic Centre, Oxford, UK. Female probands were also identified from a cohort of unselected volunteer women taking part in an unrelated survey of LS BMD at the Nuffield Orthopaedic Centre, Oxford, UK. Further male probands were recruited from men with atraumatic hip fracture presenting to the Accident and Emergency Department, John Radcliffe Hospital, Oxford, UK from January 1994 to December 1995, who were reviewed clinically with measurement of BMD.

All first-degree relatives of the probands were invited to participate in this study. When a first-degree relative of the proband was found to have low BMD, recruitment was extended to their first-degree relatives (i.e. second-degree relatives of the proband).

Participants had BMD measured at LS and FN by DXA using a QDR 1000W densitometer (Hologic Inc., Waltham, MA, USA) and were screened for secondary causes of osteoporosis by questionnaire, physical examination and biochemical screening (full blood count, erythrocyte sedimentation rate, serum urea, creatinine, liver function tests, calcium, phosphate, thyroxine, thyroid stimulating hormone, testosterone, sex hormone binding globulin, follicle-stimulating hormone, luteinising hormone, estradiol,

progesterone and prolactin). Individuals with secondary causes of osteoporosis (including corticosteroid use (≥7.5 mg of prednisolone or equivalent per day for ≥ 6 months), alcohol excess (more than 21 units per week for males or 14 units per week for females (one unit being the amount of alcoholic beverage containing 10g of ethanol)), chronic renal failure, pituitary disease, hyperparathyroidism, thyrotoxicosis, anorexia nervosa, prolonged immobilisation, malabsorption, or neoplasia) had their BMD values excluded from the study, though their DNA may have been used to help linkage analysis. Absolute BMD scores were corrected for age and sex using the Hologic US White Hip and Lumbar Spine reference data (Kelly 1990) and expressed as z-scores.

Ethical approval for the study was granted by the Central Oxford Research Ethics Committee, and all subjects gave written informed consent.

3.3 Statistical Methods

3.3.1 Descriptive Statistics

Descriptive statistics of the cohort were analysed using the Data Analysis pack of Microsoft® Excel programme (Microsoft Corporation, USA). Comparisons with the normal population were made using the one-sample t-test. Comparisons between different groups were made using the normal (or *z*) test, and all p-values quoted are two-tailed.

3.3.2 Sibling Recurrence Risk Ratio

The sibling recurrence risk ratio is often used as a measure of the genetic effect upon a trait. However, it is a measure of familiality rather than heritability *per se*, as the sibling recurrence risk ratio may be increased by increased sharing of either genes or environment by family members. The sibling recurrence risk ratio (λ_S) is calculated as:

λ_S = (prevalence of disease in siblings)/(prevalence of disease in the general population).

3.3.3 Heritability

As discussed in Chapter 1, heritability is the proportion of variance of a trait under genetic control, and may have both additive and dominance components. Heritability was calculated using the programmes Sequential Oligogenic Linkage Analysis Routines (SOLAR) (Almasy et al. 1998) and Pedigree Analysis Package (PAP) (Hasstedt 1994). Both PAP and SOLAR estimate heritability by variance components methods. The assumptions made in these analyses are: multivariate normal distribution, no epistasis, and no gene-environment interaction or correlation.

3.3.4 Correlations of BMD

Correlations of BMD between relative pairs were modelled using PAP. Comparisons of correlations were made using the normal (or *z*) test.

3.3.5 Partitioning of Variance

To assess for the effect of dominance variance, the overall correlation of BMD for parent-offspring pairs was compared with overall sibling-sibling pair correlation of BMD.

As discussed in the introduction, siblings can share 0, 1 or 2 alleles identical by descent, with probabilities of 25%, 50% and 25% respectively. Thus on average the overall allele sharing of siblings IBD is 50%. Parents and offspring have 50% allele sharing IBD absolutely, as offspring inherit 1 allele from each parent. Dominance variance can only be assessed when 2 alleles are shared IBD, as it is only present where there is interaction between alleles. Additive variance (the variance introduced due to the individual effect of an allele) is assessed where there is sharing of (only) one allele IBD. Sibling-sibling correlations therefore consist of both additive and dominance variance ($0.5\ V_A + 0.25\ V_D$), whilst parent-offspring correlations only consist of additive variance ($0.5\ V_A$).

3.4 Results

3.4.1 Descriptive Statistics of Probands

147 probands had BMD measured at LS, with mean z-score of –2.23, standard deviation (SD) 0.76 and range –4.31 to 1.30.

146 probands had BMD measured at FN with mean z-score of –1.80, SD 0.74, and range –3.82 to 0.62.

The mean age of the probands was 51.9 years (range 23.6 to 77.3 years).

3.4.1.1 Male probands

Of the 45 male probands, 10 probands had z-score < –2.0 at both LS and FN, 24 probands had z-score < –2.0 at LS only, 3 probands had z-score < –2.0 at FN only, and 8 had z-scores higher than –2.0 at both sites, although all these 8 had z-scores below –1.79, corresponding to the lowest 3.67% of the population (presented in Table 3.4.1.1).

Table 3.4.1.1: Entry Criteria for Male Probands

BMD < –2.0	**Number**
Both LS and FN	10
LS only	24
FN only	3
Neither	8

For BMD at LS, the mean z-score was –2.59, SD 0.73 and range –4.31 to –1.56.

For BMD at FN, the mean z-score was –1.68, SD 0.72, and range –3.25 to 0.62.

The mean age of male probands was 53.4 years, with range 25.4 to 77.3 years.

3.4.1.2 Female probands

Of the 102 female probands, 20 probands had z-score < –2.0 at both LS and FN, 47 probands had z-score < –2.0 at LS only, 25 probands had z-score < –2.0 at FN only and

10 had z-scores higher than –2.0 at both sites, although all these had z-scores below –1.81, corresponding to the lowest 3.59% of the population (presented in Table 3.4.1.2).

Table 3.4.1.2: Entry Criteria for Female Probands

BMD < –2.0	**Number**
Both LS and FN	20
LS only	47
FN only	25
Neither	10

For BMD at LS, the mean z-score was –2.07, with SD 0.72 and range –3.67 to 1.30.

For BMD at FN, the mean z-score was –1.85, with SD 0.75, and range –3.82 to 0.33.

The mean age of female probands was 51.4 years, range 23.6 to 76.3 years.

3.4.2 Comparisons Between Male and Female Probands

As shown in Table 3.4.2, male probands had significantly lower BMD at LS compared with female probands (–2.60 vs. –2.07, $p = 0.0001$). There was no significant difference at FN between male and female probands (–1.68 vs. –1.85, $p = 0.2$). There was no significant difference in age of probands ($p = 0.41$).

There was a significant difference in the number of probands recruited according to FN or LS ($\chi^2 = 10.8$ with 3 degrees of freedom, $p < 0.025$). This was despite the recruitment

of some female probands through the voluntary survey of BMD, which only measured BMD at LS.

Table 3.4.2: Mean BMD of Probands

Probands	LS BMD	FN BMD
All	–2.23	–1.80
Male probands	–2.59	–1.68
Female probands	–2.07	–1.85

3.4.3 Descriptive Statistics and Comparisons of BMD of Siblings

For all siblings who took part in the study:

188 siblings had BMD measured at LS, with mean z-score of –0.84, SD 1.16, and range –3.75 to 2.66. 184 siblings had BMD measured at FN, with mean z-score of –0.82, SD 0.99, and range –3.69 to 1.92. The mean BMD at both LS and FN was significantly different from the normal population (defined by the Hologic normative database), with $p = 2.6 \times 10^{-19}$ at LS and $p = 3.6 \times 10^{-23}$ at FN (presented in Diagrams 3.4.3.1 and 3.4.3.2).

The siblings of male probands had mean BMD of –0.95 at LS and –0.69 at FN.

The siblings of female probands had mean BMD of –0.80 at LS and –0.88 at FN.

There was no significant difference in mean BMD of siblings of differing gender probands ($p = 0.44$ for comparison of BMD at LS, $p = 0.28$ for comparison of BMD at FN).

Diagram 3.4.3.1: LS BMD of Siblings

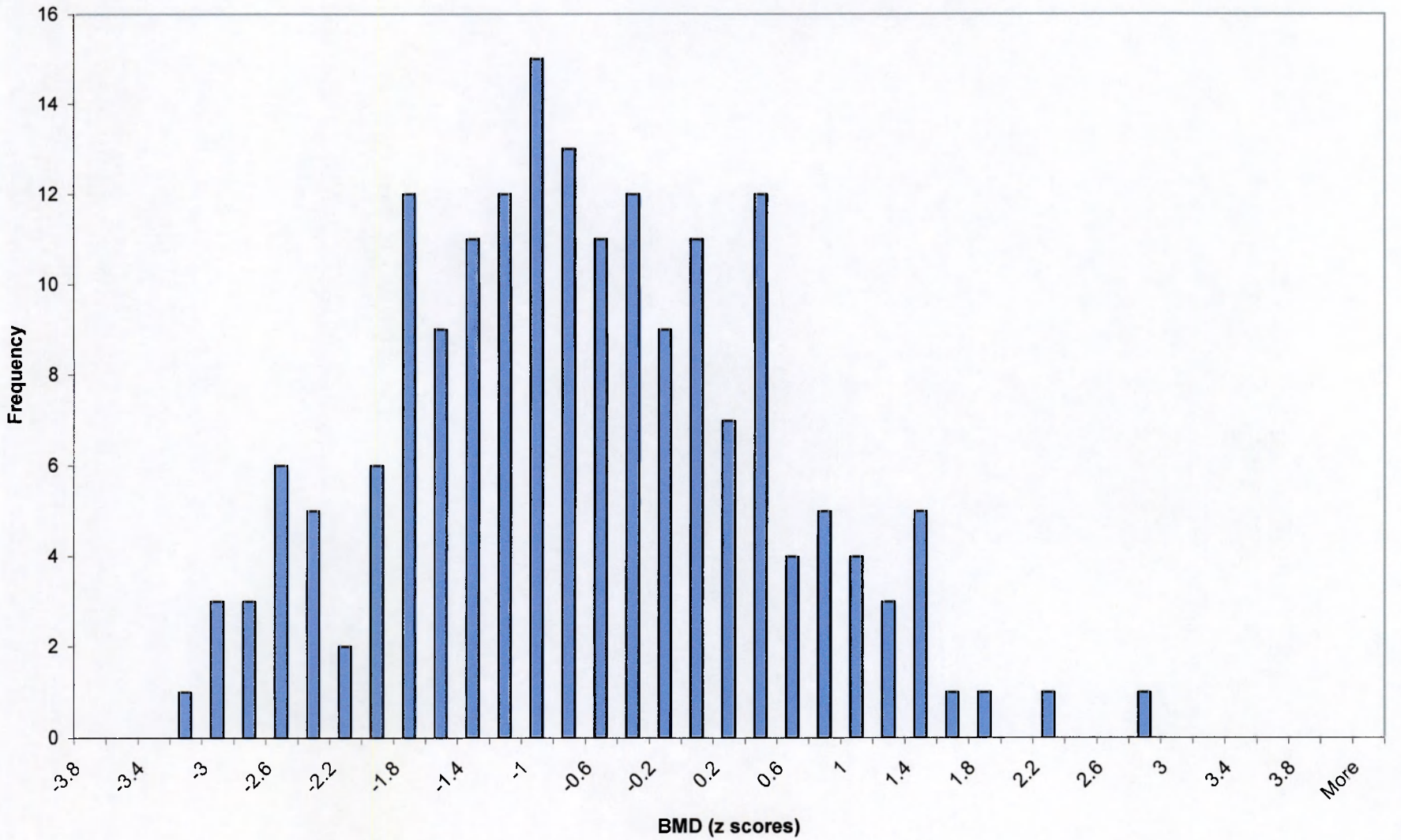


Diagram 3.4.3.2: FN BMD of Siblings

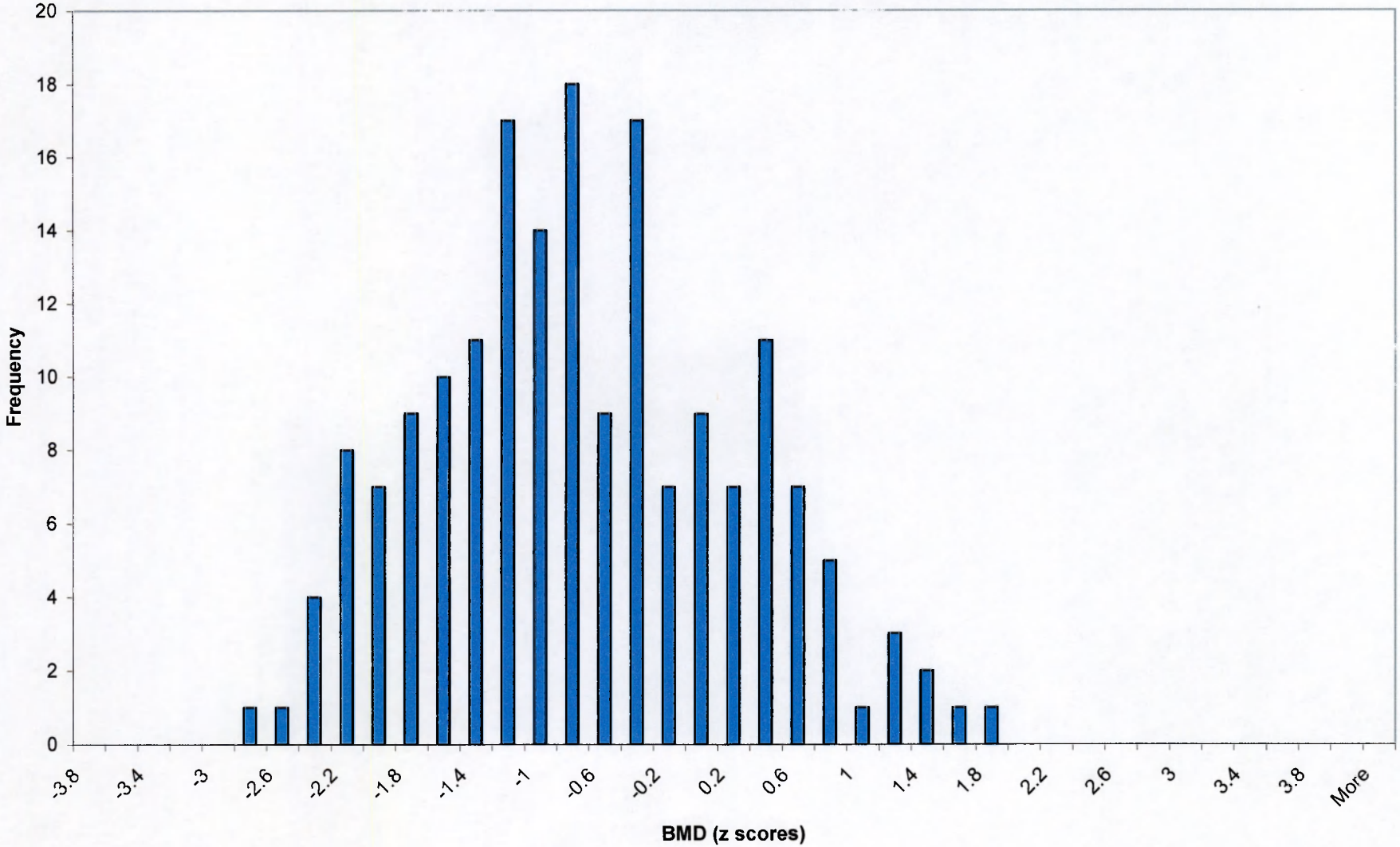


Diagram 3.4.3.3: LS BMD Comparison of Brothers and Sisters

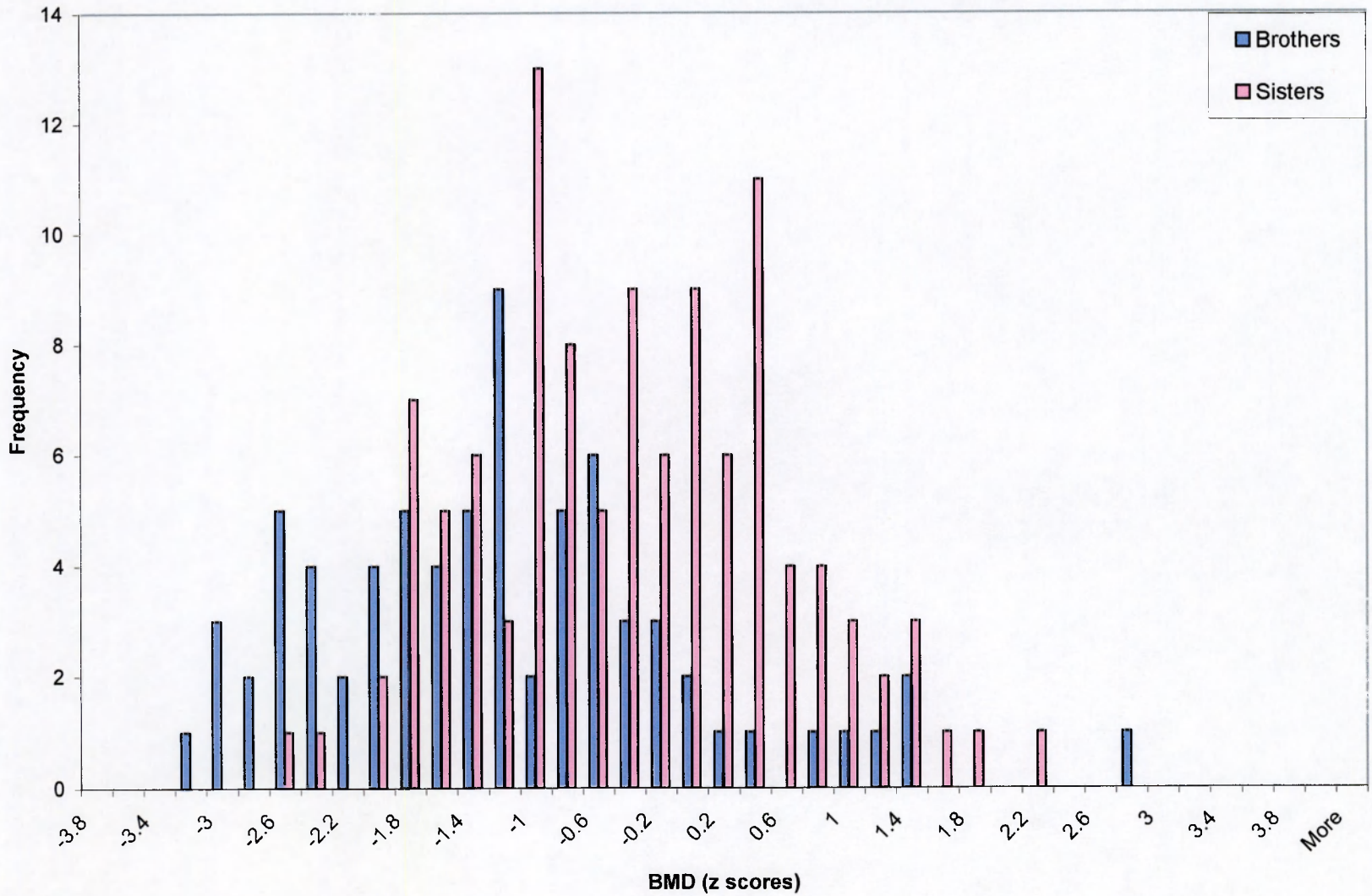
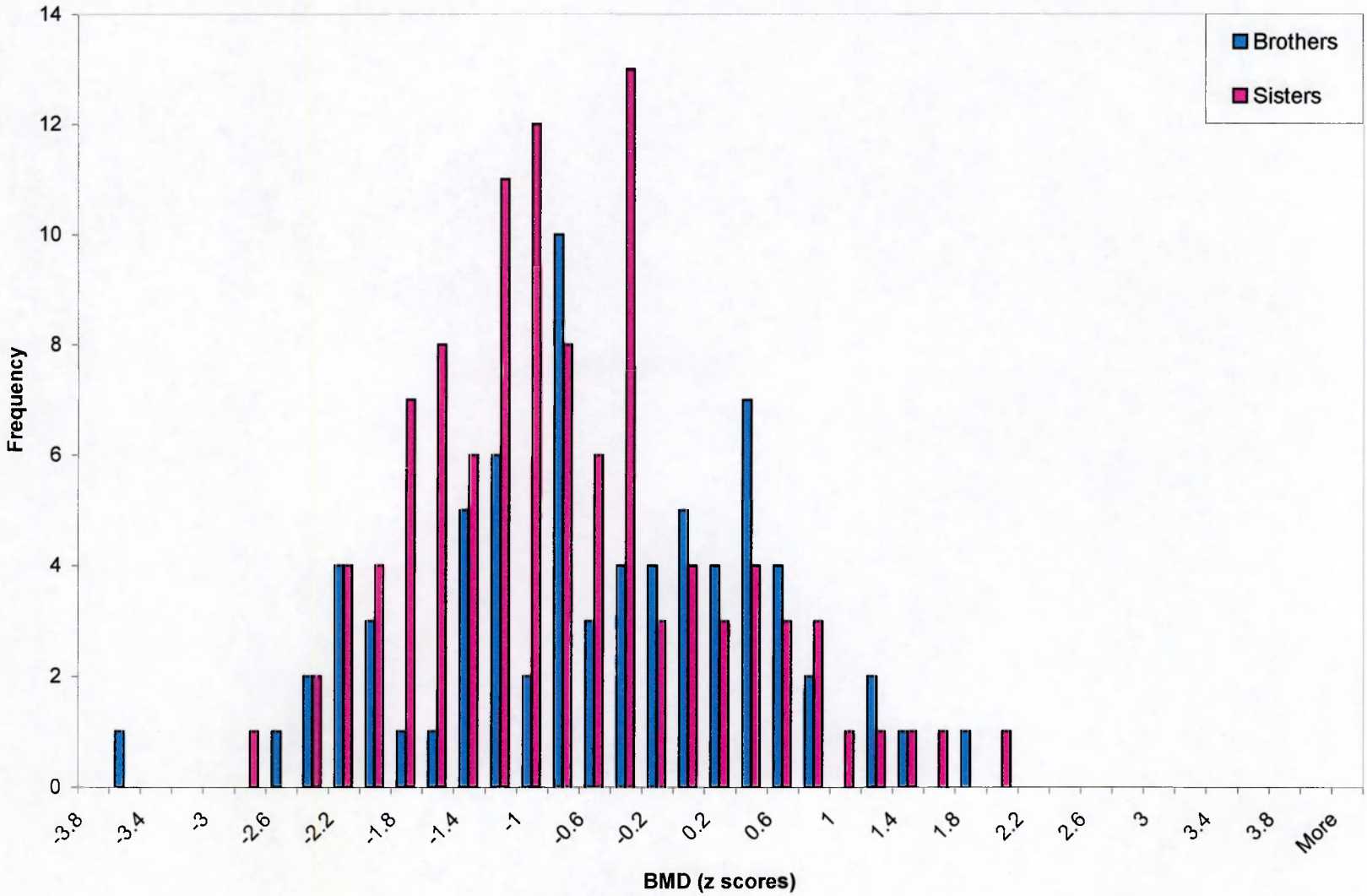


Diagram 3.4.3.4: FN BMD Comparison of Brothers and Sisters



However, when looking at the siblings according to their gender irrespective of the proband's gender, male siblings were significantly lower at LS compared with female siblings (–1.35 vs. –0.51, p = 0.0000005). There was no significant difference between male and female siblings at FN (–0.72 vs. –0.89, p = 0.25) (presented in Diagrams 3.4.3.3 and 3.4.3.4).

Table 3.4.3.1: BMD according to gender of siblings

	BMD at LS	**BMD at FN**
Brothers	–1.35	–0.72
Sisters	–0.51	–0.89
p-value	0.0000005	0.25

To see if this was due to bias arising from the differences in BMD at LS and FN between male and female probands, the siblings were subdivided into gender for each gender group of proband.

For the siblings of male probands, brothers had lower BMD at LS compared with sisters (–1.48 vs. –0.20, p<0.00002). At FN there was no significant difference between brothers and sisters (–0.63 vs. –0.78, p = 0.61) (presented in Table 3.4.3.2).

Table 3.4.3.2: Siblings of Male Probands

	BMD at LS	BMD at FN
Brothers	–1.48	–0.63
Sisters	–0.20	–0.78
p-value	<0.00002	0.61

For the siblings of female probands, the same pattern was evident. Brothers had significantly lower BMD at LS compared with sisters (–1.25 vs. –0.58, p = 0.0024), whereas there was no significant difference at FN (–0.78 vs. –0.92, p = 0.45) (presented in Table 3.4.3.3).

Table 3.4.3.3: Siblings of Female Probands

	BMD at LS	BMD at FN
Brothers	–1.25	–0.78
Sisters	–0.58	–0.92
p-value	0.0024	0.45

There was no significant difference between the brothers of male probands compared with brothers of female probands at either LS or FN. Similarly, there was no significant difference between the sisters of male probands compared with the sisters of female probands at either site (presented in Tables 3.4.3.4 and 3.4.3.5).

Table 3.4.3.4: BMD in Brothers according to Proband Gender

	BMD at LS	BMD at FN
Male proband	–1.48	–0.63
Female proband	–1.25	–0.78
p-value	0.39	0.55

Table 3.4.3.5: BMD in Sisters according to Proband Gender

	BMD at LS	BMD at FN
Male proband	–0.20	–0.78
Female proband	–0.58	–0.92
p-value	0.13	0.54

There was significant differential recruitment of siblings according to gender. Overall more sisters took part than brothers (113/188 vs. 75/188). For male probands, more brothers were recruited than sisters although this was not significant ($p = 0.13$ using binomial test and assuming equal gender distribution in siblings). For female probands significantly more sisters were recruited than brothers ($p = 0.000032$). Overall χ^2 test for recruitment of brothers and sisters showed significant differences with corrected χ^2 of 9.59, $p = 9.77 \times 10^{-4}$. The recruitment of siblings according to gender is presented in Table 3.4.3.6.

Table 3.4.3.6: Sibling Recruitment According to Gender

	Male proband	Female proband	Total
Brothers	31	44	75
Sisters	22	91	113
Total	53	135	188

There was a significant difference between LS and FN z-scores of siblings according to proband recruitment through either LS or FN. Siblings of probands recruited through low BMD only at FN (z-score < -2) had significantly lower BMD at FN than did siblings of probands with low BMD only at LS (–1.33 vs. –0.61, $p = 0.00005$). There was no significant difference at LS (–0.79 vs. –0.77). This is presented in Table 3.4.3.7.

Table 3.4.3.7: BMD in Siblings at LS and FN According to Proband Recruitment Site

BMD site	**Proband with low LS BMD only**	**Proband with low FN BMD only**	**p-value**
LS	–0.77	–0.79	0.93
FN	–0.61	–1.33	0.00005

3.4.4 Sibling Recurrence Risk Ratio

The sibling recurrence risk ratio (λ_S) of low BMD defined as a z-score < -2.0 was calculated at both LS and FN.

Of 189 siblings with BMD measured at LS, 27 had a z-score < –2.0, giving λ_S of low BMD at LS of 6.26, with confidence limits 4.08 to 8.46. Of note, 22 % of siblings fulfilled the WHO definition of osteoporosis (t-score < –2.5) and 58% fulfilled the WHO definition of osteopaenia (t-score < –1.0).

Of 184 siblings with BMD measured at FN, 22 had a z-score < –2.0, giving λ_S of low BMD at FN of 5.24, with confidence limits 3.19 to 7.30. 35% fulfilled the WHO definition of osteoporosis and 66% fulfilled the WHO definition of osteopaenia.

40 known siblings did not take part. Two siblings gave blood samples but did not have BMD measured. Two siblings had their BMD result excluded due to the presence of other disease(s) affecting bone. Nine siblings did not take part because of prohibitive distance. The remainder (27) either refused to take part or the reason for nonparticipation was not known.

3.4.5 Descriptive Statistics of Relatives and Comparison of BMD

For all relatives who took part in the study:

596 relatives had BMD measured at LS, with mean z-score of –0.7402, SD 1.217, and range –4.58 to 5.44.

582 relatives had BMD measured at FN, with mean z-score of –0.8278, SD 1.082, and range –3.83 to 5.64.

There was no difference in BMD of relatives according to proband gender, at either LS or FN (presented in Table 3.4.5.1).

Table 3.4.5.1: BMD of Relatives According to Proband Gender

Proband Gender	BMD at LS	BMD at FN
Relatives of male probands	–0.857	–0.781
Relatives of female probands	–0.699	–0.844
p-value	0.186	0.552

Overall, male relatives had lower BMD at LS compared with female relatives (–0.976 vs. –0.576, p = 0.0001). There was a non-significant trend for lower BMD in female relatives compared with male relatives at FN (–0.895 vs. –0.732, p = 0.073) (presented in Table 3.4.5.2).

Table 3.4.5.2: BMD of Male Relatives Compared with Female Relatives

Gender of Relatives	BMD at LS	BMD at FN
Male relatives	–0.976	–0.732
Female relatives	–0.576	–0.895
p-value	0.0001	0.073

The relatives were subdivided according to proband gender. At LS, male relatives of the male probands had significantly lower BMD than female relatives (–1.214 vs. –0.466, p =

0.0002). There was no significant difference at FN (–0.69 vs. –0.8817, p = 0.31) (presented in Table 3.4.5.3).

Table 3.4.5.3: BMD of Relatives of Male Probands

Relatives of male probands	BMD at LS	BMD at FN
Male relatives	–1.214	–0.690
Female relatives	–0.466	–0.882
p-value	0.0002	0.31

Again, the same pattern was observed with relatives of female probands, with lower BMD at LS observed in male relatives compared with female relatives (–0.858 vs. –0.605, p = 0.038). A non-significant trend for lower BMD at FN in female relatives compared with male relatives was observed (–0.898 vs. –0.752, p = 0.16) (presented in Table 3.4.5.4).

Table 3.4.5.4: BMD in Relatives of Female Probands

Relatives of female probands	BMD at LS	BMD at FN
Male relatives	–0.858	–0.752
Female relatives	–0.605	–0.898
p-value	0.038	0.16

At LS, male relatives of male probands had significantly lower BMD than male relatives of female probands (p = 0.036). This may be due to the bias introduced by lower BMD at

LS of the male probands. There was no other significant difference between male and female relatives according to proband gender (presented in Tables 3.4.5.5 and 3.4.5.6).

Table 3.4.5.5: BMD in Male Relatives According to Proband Gender

	BMD at LS	**BMD at FN**
Male proband	–1.214	–0.690
Female proband	–0.858	–0.752
p-value	0.036	0.70

Table 3.4.5.6: BMD in Female Relatives According to Proband Gender

	BMD at LS	**BMD at FN**
Male proband	–0.466	–0.882
Female proband	–0.605	–0.898
p-value	0.40	0.90

For the 233 young relatives (aged 35 years or less) mean z-score at LS was –0.962 (SD 1.01, range –4.58 to 2.38). At FN mean z-score was –1.09 (SD 1.09 with range –3.83 to 2.74). These were significantly lower than the general population ($p < 10^{-35}$ for both comparisons).

3.4.6 Correlations between Relative Pairs

Correlations of BMD adjusted for age and sex (i.e. correlation of z-scores) for relative pairs were assessed using PAP with mean and standard error (s.e.) shown in Table

3.4.6.1. The analysis was repeated using height as a covariate as a proxy for bone size, with results shown in Table 3.4.6.2. Statistical comparisons of these data are presented in Tables 3.4.6.3 to 3.4.6.6.

Abbreviations used in this section:

MD Mother-daughter

MS Mother-son

FD Father-daughter

FS Father-son

SS Sister-sister

SB Sister-brother

BB Brother-brother

Table 3.4.6.1: Correlation of BMD (unadjusted for measures of bone size)

Relative pair	**Correlation at LS (s.e.)**	**Correlation at FN (s.e.)**
MD	0.137 (0.065)	0.247 (0.063)
MS	0.173 (0.067)	0.166 (0.078)
FD	0.166 (0.060)	0.067 (0.076)
FS	0.260 (0.068)	0.0625 (0.089)
SS	0.207 (0.081)	0.293 (0.071)
SB	0.153 (0.079)	0.198 (0.073)
BB	0.359 (0.076)	0.207 (0.100)

Table 3.4.6.2: Correlation of BMD (adjusted for height)

Relative pair	Correlation at LS (s.e.)	Correlation at FN (s.e.)
MD	0.133 (0.066)	0.225 (0.038)
MS	0.169 (0.069)	0.169 (0.038)
FD	0.166 (0.060)	0.100 (0.038)
FS	0.261 (0.069)	0.0660 (0.039)
SS	0.204 (0.082)	0.269 (0.039)
SB	0.150 (0.080)	0.226 (0.039)
BB	0.361 (0.076)	0.244 (0.038)

A consistent trend was noted that all male-male comparisons were greater at LS than at FN, whilst all female-female comparisons were greater at FN than at LS. Further, at LS male-male comparisons were higher than female-female comparisons, whilst at FN female-female comparisons were greater than male-male comparisons. The comparisons are shown in Tables 3.4.6.3 - 6, with p-values.

Table 3.4.6.3: Correlations of BMD (unadjusted for bone size) Between Relative Pairs

Relative pair	Correlation at LS	p-value	Correlation at FN	p-value
BB vs. SS	0.359 vs. 0.207	0.17	0.207 vs. 0.293	0.48
MD vs. FS	0.137 vs. 0.260	0.19	0.247 vs. 0.0625	0.09
MD vs. MS	0.137 vs. 0.173	0.70	0.247 vs. 0.166	0.42
FD vs. FS	0.166 vs. 0.260	0.30	0.0667 vs. 0.0625	0.97
MS vs. FS	0.173 vs. 0.260	0.36	0.166 vs. 0.0625	0.38
MD vs. FD	0.137 vs. 0.166	0.74	0.247 vs. 0.0667	0.07

Table 3.4.6.4: Comparison of BMD Correlation (unadjusted for bone size) at LS and FN

Relative pair	Correlation of BMD at LS vs. FN	p-value
Brother-brother	0.359 vs. 0.207	0.23
Sister-sister	0.207 vs. 0.293	0.42
Mother-daughter	0.137 vs. 0.247	0.22
Father-son	0.260 vs. 0.0625	0.08

Table 3.4.6.5: Correlations of BMD (adjusted for height) Between Relative Pairs

Relative pair	Correlation at LS	p-value	Correlation at FN	p-value
BB vs. SS	0.361 vs. 0.204	0.16	0.244 vs. 0.269	0.65
MD vs. FS	0.133 vs. 0.261	0.18	0.225 vs. 0.0660	0.0035
MD vs. MS	0.133 vs. 0.169	0.7	0.225 vs.0.168	0.3
FD vs. FS	0.166 vs. 0.261	0.29	0.100 vs.0.0660	0.53
MS vs. FS	0.169 vs. 0.261	0.34	0.168 vs. 0.0660	0.06
MD vs. FD	0.133 vs. 0.166	0.71	0.225 vs. 0.100	0.02

Table 3.4.6.6: Comparison of BMD (adjusted for height) at LS and FN

Relative pair	Correlation of BMD at LS vs. FN	p-value
Brother-brother	0.361 vs. 0.244	0.17
Sister-sister	0.204 vs. 0.269	0.47
Mother-daughter	0.133 vs.0.225	0.23
Father-son	0.261 vs. 0.0660	0.014

3.4.7 Heritability

Using PAP, heritability of BMD at LS was 0.453, increasing to 0.484 when height and weight were added in as covariates. Heritability of BMD at FN was 0.424, increasing to 0.482 when height and weight were added in as covariates.

Using SOLAR, the heritability of BMD at LS was 0.639 and at FN was 0.432, with BMI used as a covariate.

Of note, correction for ascertainment had a marked effect upon heritability estimates. Using a subset of 710 individuals and disregarding ascertainment, heritability estimates for BMD were 0.184 at LS and 0.198 at FN. Correcting for ascertainment bias resulted in substantial increases in heritability estimates to 0.453 at LS and 0.424 at FN.

3.4.8 Partitioning of Variance

Initial work using 710 individuals showed overall parent-offspring correlation at LS of 0.222 (s.e. 0.041) with sibling correlation of 0.267 (s.e.0.055) (no significant difference). At FN, parent-offspring correlation was 0.186 (s.e. 0.033) whilst sibling correlation was 0.287 (s.e. 0.039). These were significantly different ($p = 0.048$) and suggested that dominance variance was present at FN.

From sibling correlation: $\frac{1}{2} V_A + \frac{1}{4} V_D = 0.287$

From parent-offspring correlation: $\frac{1}{2} V_A = 0.186$ and total $V_A = 0.372$

Therefore: $\frac{1}{4} V_D = 0.101$ and total $V_D = 0.404$

As a proportion of total genetic variance $V_D = V_D/(V_A + V_D)$

$= 0.404/(0.404+0.372) = 52.06\%$.

Subsequent work was performed using 833 individuals. Using unadjusted z-scores, overall parent-offspring correlation at LS was 0.186 (s.e. 0.0366) compared with sibling-sibling correlation of 0.225 (s.e. 0.0502) ($p = 0.53$). At FN, parent-offspring correlation was 0.151 (s.e. 0.0408) compared with sibling-sibling correlation of 0.239 (s.e. 0.0513) ($p = 0.18$). Thus although the same trend was present there was no longer a significant difference.

When height was included as a covariant as a surrogate correction for bone size, at LS there was no significant difference between parent-offspring correlation and sibling-sibling correlation (0.187 vs. 0.233; $p = 0.46$). At FN, parent-offspring correlation was 0.150 (s.e. 0.042), compared with sibling-sibling correlation of 0.252 (s.e. 0.051) ($p = 0.12$), demonstrating a similar, though non-significant, trend.

3.5 Discussion

This chapter presents the genetic epidemiology from a family cohort collected for the purpose of linkage studies into the genetics of osteoporosis.

As detailed above, probands for this study were selected with an extremely low BMD relative to an age- and sex-matched cohort, using the Hologic QDR-1000 manufacturer's normative database (Hologic Inc., Waltham, MA, USA). Ascertainment schemes where

probands are selected for extreme quantitative trait values are more powerful for gene detection than randomly recruited families. Using unselected sibling pairs, only loci contributing a large amount (around 50%) to the total heritability of a trait will be detectable by the Haseman-Elston allele-sharing method in a 'reasonably sized' sample, and thousands of sib pairs would be needed to detect linkage to loci contributing less than this, even using multipoint analysis (Blackwelder and Elston, quoted in (Risch et al. 1995). Use of sibling pairs selected through a single proband with an extreme trait value (e.g. 5-10% of the tail of distribution of the quantitative trait) dramatically increases power both for two-point (Cardon et al. 1994; Carey et al. 1991) and multipoint (Cardon et al. 1995) linkage analysis. Carey and colleagues showed that using sibling pairs sampled through one proband with a trait value in the extreme 5% of the tail resulted in an order of magnitude increase in power compared with using unselected siblings, such that loci responsible for 10-20% of the phenotypic variance of a trait could be detected with 80% power in sample sizes potentially as low as 73 sib pairs (depending on dominance and allele frequencies) (Carey et al. 1991). Risch and Zhang further demonstrated that a 'double proband' approach, using siblings either extremely concordant (for high or low values) or extremely discordant, was in general even more powerful, with greatest power obtained using extremely discordant sibling pairs (Risch et al. 1995). However, this may be dependent upon allele frequencies and the underlying genetic model (Allison et al. 1998). Further, finding extremely discordant sibling pairs can be very difficult and power may have to be balanced against the expense of ascertainment (Gu et al. 1997; Gu et al. 1996). The effect of ascertainment bias (in choosing probands with extreme trait values) upon heritability estimates must also be

considered. The comparison of heritability estimates obtained with and without correction for ascertainment (Section 3.4.7) demonstrates that failure to correct for ascertainment bias results in loss of power to detect linkage (Fisher et al. 1999; Marlow 2001).

For this study, families were recruited through a single affected proband (BMD z-score < –2.0, corresponding to the lowest 2.275% of the age- and sex-matched population distribution, in addition to having osteoporosis as defined by WHO (Kanis et al. 1994)). The greater power of this ascertainment scheme was, however, at the cost of increased difficulty recruiting families.

The original Hologic US White Hip and LS reference database, compiled using a North American Caucasian population, were used to define z-scores for both men and women. Previous studies of comparisons between Hologic and other manufacturers of bone densitometers (Lunar Corporation, Madison, WI, USA and Norland Medical Systems Inc., Fort Atkinson, WI, USA) had shown that although measurement of absolute BMD (in g/cm^2) was highly correlated between machines, the z- and t-scores varied according to the manufacturer's normative database. Results using the Hologic machines consistently resulted in lower t-scores at FN, although there was no difference in LS t-scores between the different machines (Faulkner et al. 1996; Pocock et al. 1992).

Hologic has subsequently altered its FN reference database, implementing the normative database established by the US National Health and Nutrition Examination Survey (NHANES III) (Looker et al. 1998). This large study measured BMD and bone mineral content using DXA of the proximal femur in a nationally representative sample of both

men and women. The mean FN BMD values for white men and women were approximately 3-5% lower than the Hologic normative database, and the SDs 26-30% higher. The difference in mean BMD applied to both young and old age groups. The difference in SD according to age varied according to the different region of interest examined. However for FN, there was no clear pattern of difference in SD with age (Looker et al. 1995). Thus use of the new database would result in both t- and z-scores changing, but the relative changes for a younger or older person should be similar. Further, although both FN and total hip BMD had the same predictive value for hip fracture, precision error of BMD at total hip was approximately half that of FN, of particular importance in follow-up scans. Hence BMD of total hip has now become the standard region of interest at this site (Chen et al. 1998).

Neither the NHANES nor the previous Hologic normative databases have been formally compared with the Oxfordshire population at both LS and FN. Several studies have suggested the importance of using locally-derived normative data, due to substantial geographic variability in BMD (Crabtree et al. 2000; Lofman et al. 2000; Simmons et al. 1995). One UK study of both volunteer women and women with possible osteoporosis did not find a difference in mean and SD of BMD at LS compared with the Hologic normative database in either group (Ryan et al. 1993). However, comparison of mean, standard error and standard deviation established using BMD from 650 Oxfordshire women volunteers and the Hologic normative database revealed substantial differences. Peak bone mass was obtained at a later age (30-40 years). Mean BMD was generally higher, and SD widened with age (personal communication, AJ Shipman and I Smith). This suggests that it would be useful to have a local database for assessment of BMD.

Unfortunately, the Oxfordshire study only measured BMD at LS, and only in women. To maintain consistency, therefore, the Hologic database was used.

The descriptive statistics presented in this chapter were based upon z-scores obtained with the old Hologic normative database. The average difference between t-scores obtained at the hip using the NHANES database and the previous Hologic database is 0.64, which substantially decreases the number of individuals with osteoporosis and osteopaenia at FN according to WHO guidelines (Chen et al. 1998). Therefore the number of siblings and relatives in this family collection who fulfilled WHO guidelines for osteoporosis (t-score < -2.5) or osteopaenia (t-score -1 to -2.5) at the hip may have been extensive. Further, there may have been excessive numbers of relatives with z-scores below -2.0, and thus the sibling recurrence risk ratio at FN may be upwardly biased. However, the results at LS were not biased.

This work is the first to define a sibling recurrence risk ratio (λ_S) for low BMD. Of note, however, λ_S is a measure of familiality rather than a measure of genetic effects *per se*, as it does not differentiate between increased prevalence of disease in siblings arising from shared enviroment or shared genes. Further, diseases with high population prevalence may have quite low λ_S despite being substantially genetically determined, i.e. having high heritability. Asthma is an example of a highly heritable disease with low λ_S, with heritability of approximately 70% yet a sibling recurrence risk ratio of 1.5 (Palmer et al. 2000). Examples of heritability estimates and λ_S for other heritable musculoskeletal diseases are shown in Table 3.5.1.

Table 3.5.1: Heritability and Sibling Recurrence Risk Ratios

Disease	**Heritibility**	**Sibling Recurrence Risk Ratio**	**Prevalence**
Rheumatoid Arthritis	53-65%[1]	14[2] 4[3]	1-3%
Ankylosing Spondylitis	95[4]	82[4]	0.1-0.5%
Osteoarthritis	27-58%[5,6]	1.9[6]	60-75%
Systemic Lupus Erythematosis	66[7]	24[7]	0.05%

[1](MacGregor et al. 2000); [2](Wordsworth 1995); [3](Rigby et al. 1998); [4](Brown et al. 2000); [5](MacGregor et al. 2000); [6](Chitnavis et al. 1997); [7](Lawrence et al. 1987).

The heritability estimates obtained with these families are in keeping with those previously reported from family studies (Deng et al. 2000; Gueguen et al. 1995; Sowers et al. 1992). Of note, Gueguen and colleagues reported that heritability of BMD was highest in young individuals, maximal at age 26 years with heritability of 84%, and Deng and colleagues reported similar high heritability of peak bone mass (Deng et al. 1999; Gueguen et al. 1995). The young relatives of this cohort (aged 35 years or less) had significantly low BMD. These results are consistent with heritability of low peak bone mass rather than of bone loss, although this was not formally compared.

The differences between BMD at LS for male and female probands (Table 3.4.2) may have arisen because of different means of recruitment. Most male probands had symptomatic osteoporosis, evidenced by fracture, wherease many of the female probands had been identified through the voluntary survey at BMD. Their participation in this survey may have been prompted by a realistic concern of personal risk of osteoporosis (for example, by having a family history of fracture). Although there was a significant difference between the numbers of male and female probands recruited with low BMD at LS or FN (or both), the bulk of the contribution to the χ^2 statistic was due to disproportionate recruitment at FN, not at LS, with a greater proportion of female probands recruited through FN compared with male probands. The use of the old database may have meant that those probands recruited through low BMD at LS had more severe osteoporosis than those recruited through FN. This would not have affected linkage results, but may have diminished power to detect genetic effects by recruitment of probands with a less extreme phenotype (see above discussion).

Brothers had consistently lower BMD at LS than sisters (Table 3.4.3.1). This may have been due to the greater number of brothers recruited through a male proband, and to the lower LS BMD of male probands compared with female probands. However, this trend was observed in brothers of both male and female probands (Tables 3.4.3.2 and 3.4.3.3). Further, there was no significant difference in BMD between brothers of male probands compared with brothers of female probands, nor between sisters of male probands compared with sisters of female probands (Table 3.4.3.4 and 3.4.3.5). Thus the low BMD at LS observed in brothers was not due to biases in either proband recruitment or the relative numbers of brothers and sisters recruited according to proband gender. A

possible explanation for this is that there are male-specific genes contributing to BMD at LS. A further possibility is that the Hologic database for LS for males gives excessively low t- and z-scores compared with the female database. Some support for a possible under-reporting of male BMD was found by Tai and colleagues who demonstrated a small gender bias in z-scores at LS but not FN (Tai et al. 2001). However, the results using all relatives did not support evidence of systematic error in the Hologic normative database.

The results presented in Table 3.4.3.7 support the concept of site-specificity of inheritance of BMD. Siblings of patients recruited through low BMD at FN had significantly lower BMD at FN than siblings of probands recruited through low BMD at LS. This would support the concept of site-specific genes in determination of BMD: that relatives of probands with severe osteoporosis at one site shared more alleles determining low BMD at this site, and thus manifest lower BMD at this site. The correlations of BMD between relative pairs (presented in Section 3.4.6) also suggested site-specific genetic effects at both LS and FN. The initial work partitioning genetic variance into its components supported evidence of dominance variance at FN but not at LS, which again is consistent with site-specific genetic effects. However, parent-offspring and sibling-sibling correlations at FN were no longer significantly different with the expanded data set. Greater numbers of families are needed for a definitive answer.

The concept of site-specific genetic effects is supported by the work of several lines of investigation. As mentioned in the introduction, although BMD is correlated between sites and BMD at one site is predictive of fracture overall, BMD of fracture patients is

frequently lowest at the site of fracture, suggesting both generalised and site-specific effects upon BMD. Seeman and colleagues measured BMD at multiple sites in premenopausal daughters of mothers with vertebral fractures and demonstrated low BMD at all sites measured; however BMD was lowest at LS, the site of maternal fracture (Seeman et al. 1989). In contrast, premenopausal daughters of mothers with hip fractures had reduced BMD at FN and femoral shaft, but normal BMD at LS (Seeman et al. 1994). Fox and colleagues also demonstrated site specificity of family history of fracture in predisposition to fracture (Fox et al. 1998), although this may be due not only to BMD but also to other shared factors predisposing to fracture (e.g. hip geometry). A study comparing shared and specific genetic effects upon BMD at different sites concluded that a substantial proportion – but not all – genetic effects on femoral neck were shared with genetic effects at lumbar spine and total body BMD, with genetic correlations between FN and LS of 0.64 and between LS and total BMD of 0.75 (Nguyen et al. 1998).

The mechanism for different genetic effects at different sites may be due to several possible mechanisms. Appendicular and axial skeletal growth are disparate (Bass et al. 1999), and thus various effects upon peak bone mass may differ at hip and spine. The relative proportions of cortical and cancellous bone differ at FN and LS. Factors exerting differential effects upon cortical and cancellous bone will therefore differ in their relative contribution to overall BMD at each site. For example, PTH is thought to have a primarily anabolic effect upon trabecular bone, whilst having a catabolic effect upon cortical sites (Bilezikian et al. 2000; Duan et al. 1999). Intermittent administration of the PTH analogue (parathyroid hormone 1-34) at doses of 20μg/day or 40μg/day increased BMD at both LS and FN; however this was considerably greater at LS (9% and 13%

respectively) than at FN (3% and 6%) (Neer et al. 2001). Another mechanism may be that gene expression induced by torsion, loading, or other physical stresses may differ at sites under such different physical stresses as LS and FN.

Further, this low BMD at LS was only manifest in male relatives of both male and female probands, not in female relatives (Table 3.4.5.3 and 3.4.5.4). Whilst this may be due to an incorrect normative male database, another possibility is gender-specific genetic effects. Other examples of heritable diseases in which there are substantial gender differences in disease manifestation include autoimmune disease such as thyroiditis, rheumatoid arthritis, systemic lupus erythematosis, and ankylosing spondylitis.

The correlations of BMD at LS and BMD of various relative pairs suggested both site-specific and gender-specific genetic effects. Male-male correlations were highest at LS compared with FN. Further, male-male correlations at LS were higher than female-female correlations at LS. Female-female correlations were higher at FN than at LS, and female-female correlations were higher than male-male correlations at FN (presented in Tables 3.4.6.1- 6). Jouanny and colleagues (Jouanny et al. 1995) demonstrated evidence of gender-specific effects upon BMD, measuring total body BMD in nuclear families. A mother's BMD was more closely correlated to her daughter's BMD whereas a father's BMD was more closely correlated to his son's BMD, with same-gender parent-child correlations being higher than cross-gender correlations (Jouanny et al. 1995). Jones and Nguyen also found evidence of gender-specific effects, with heritability estimates obtained using mother-daughter pairs significantly higher than those obtained using mother-son pairs (Jones et al. 2000). Other authors have also found greater correlation

between mother-daughter pairs compared with mother-son pairs (McKay et al. 1994). Of note, given the greater female-female correlations at femoral neck (presented in Tables 3.4.6.1-6), Jones and Nguyen also found mother-daughter correlations to be higher at FN (0.4) than at LS (0.3) or total BMD (0.22), although these correlations were not significantly different (Jones et al. 2000). Fox and colleagues found that the risk of hip fracture increased with maternal (but not paternal) history of hip fracture (Fox et al. 1998). The Rancho Bernardo study found that low BMD at LS in both sons and daughters was associated with a paternal history of osteoporosis, and low BMD at FN in sons (but not daughters) was associated with a maternal history of osteoporosis. However, this latter study did not measure parental BMD (Soroko et al. 1994).

Comparisons between relatives were performed both with and without correction of BMD for bone size. As discussed in Chapters 1 and 4, regression of BMD on height, weight or BMI results in a bone density measurement less dependent upon bone size than is uncorrected areal BMD, and allows approximation of a calculated volumetric bone density (Compston 1995). However, it is not a true volumetric measurement. Volumetric DXA or quantitative CT scanning of relatives would have provided true volumetric measurement removing bias of bone size between the sexes. This was not possible in this study and quantitative CT scanning also involves substantially greater exposure to radiation. Adjusting BMD for bone size is not usually considered in clinical practice where both BMD and bone size may be of clinical relevance (see Chapter 1). When comparing BMD between family members (particularly in linkage studies) or in the general population (for association studies), the bias introduced by bone size must be

considered, particularly when comparing BMD between men and women (Faulkner et al. 1995).

Height was used as a surrogate measure of bone size and included as a covariate when assessing BMD correlations with PAP. The covariate of weight was unable to be accurately assessed using the programme PAP. When added in, the mean BMD for the most likely model at which PAP coalesced was positive, which was known to be incorrect from the previous descriptive statistics. There are two possible reasons for this. The study may not have had adequate power to accurately assess the correlation between BMD and weight, particularly where multiple other within-family correlations were being simultaneously assessed. With weight included as a covariate, PAP models where the mean BMD was fixed at a range of negative values had similar likelihoods to those where PAP was allowed to estimate the mean BMD freely, despite the difference in sign of the mean BMD. An alternative explanation was that the effect of current weight upon BMD was not great, given the variability of weight over an individual's lifespan. However, this was not supported by a recent report from the Framingham study indicating that both low weight and recent weight loss (though not gain) affected BMD in both men and women (Hannan et al. 2000).

It is possible that the comparisons between relative pair BMD correlation at the different sites were biased by using the old database (see Tables 3.4.6.4 and 3.4.6.6). Assuming that correlations at FN would have decreased due to the smaller SD, adjustment for this bias may have decreased correlations at FN. This may have reduced the trend for female-female correlations to be higher at FN than at LS, but it would have increased the

difference between male-male comparisons at LS compared with FN. Additionally, NHANES III did not report a gender difference in the changes to mean and SD for BMD.

In conclusion, the results of this chapter have substantial implications for the design and interpretation of gene-mapping projects in osteoporosis. Gender-specific effects may only be manifest in analysis restricted to one gender. Genetic determinants of BMD at various regions of interest may be different and need to be considered separately. Genetic studies utilising total BMD may blur these site-specific effects. Although the selection of probands with extreme BMD improves power to detect linkage, ascertainment bias must be considered to prevent artificially low estimates of heritability, with their knock-on effects upon linkage analysis. Finally, screening relatives (particularly siblings) of patients with osteoporosis for low BMD may prove a cost-effective way of preventing fracture.

Chapter 4: A Candidate Gene Linkage Study in Families with Osteoporosis

4.1 Introduction

Studies of the genetic epidemiology of BMD in twins and families have shown that BMD is a highly heritable trait. Although several genes have been studied for association with BMD, very few linkage studies have been performed.

The use of candidate gene linkage and association studies to identify disease-causing genes has been most successful in monogenic disorders (e.g. Duchenne muscular dystrophy). Linkage analysis has also been successful in identifying genes underlying a subset of cases in otherwise more complex disease groups, where inheritance appears more consistent with a Mendelian model (e.g. BRCA-1 in some cases of familial early onset breast and ovarian cancer (Hall et al. 1992) and presenilin-2 in Volga Germans affected with Alzheimer's disease (Rogaev et al. 1995)). However, most complex genetic diseases, such as diabetes mellitus types 1 and 2, asthma, Alzheimer's disease and osteoporosis, arise from the action of multiple genes and their interaction with each other (epistasis) and the environment. The identification of genes in complex genetic disorders is therefore considerably more difficult. This is borne out by the paucity of genes identified in these complex disorders. To date, only the insulin gene in type 1 Diabetes Mellitus (Bell et al. 1984), Apolipoprotein E4 in late onset familial Alzheimer's Disease (Corder et al. 1993; Myers et al. 1996; Saunders et al. 1993) and Calpain-10/NIDDM 1 in type 2 Diabetes Mellitus (Horikawa et al. 2000) have been identified as contributing to population susceptibility to these common conditions, despite the plethora of whole genome screens in families with these (Concannon et al. 1998; Cox et al. 1999; Davies et

al. 1994; Hanis et al. 1996; Hashimoto et al. 1994; Pericak-Vance et al. 1991) and other inherited complex diseases. Notably, these genes were finally identified through candidate gene association studies rather than linkage screens.

For complex traits, linkage studies can only identify broad chromosomal areas that might contain a possible disease-causing gene. Although at a population level linkage disequilibrium extends only a short distance (possibly as little as 3kB (Kruglyak 1999)), within a family there are relatively few meioses occurring in the few generations studied, and thus within each family linkage will extend much further. This is exploited in planning marker density in whole genome scans, such that approximately 300 markers are sufficient to screen the whole genome. Once areas of interest have been identified, suitable candidates can be screened by positional cloning and linkage disequilibrium methods. However, genes exerting only a small or modest effect upon disease may be missed by the initial linkage screen. Greater marker density would increase the power of the study but such an approach is inefficient and expensive.

In the first part of this chapter, a large candidate gene linkage study in families with osteoporosis is presented. By only examining candidate genes, markers could be selected lying closer to the genes of interest than would probably occur in a full genome screen. This increases the power to detect linkage such that even genes exerting only a modest effect upon BMD may be detectable.

4.2 Materials and Methods

4.2.1 Family members

For proband and family ascertainment see Chapter 3. 115 families participated in this linkage study, comprising 613 individuals. BMD data were available on 572 individuals (236 males (41%), 336 females (59%)), of whom 165 (70 males (42%), 95 females (58%)) had a BMD z-score <–2.0 for either the FN (75 (45%)) or LS (121 (73%)). For 41 individuals, only DNA samples were available.

The mean age of participants was 50 years (range 16-90 years). The mean FN BMD z-score was –0.93 (range –3.71 to 4.97, n = 558) and LS BMD z-score was –1.00 (range –4.55 to 5.03, n = 568).

4.2.2. Candidate gene identification

Candidate gene genetic and physical locations were established from the literature and publicly available data bases (including http://www.gdb.org/gdb/gdbtop.html, http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/maps.cgi, http://www.ncbi.nlm.nih.gov/, http://www3.ncbi.nlm.nih.gov/Omim/) (see table 4.2.2).

Table 4.2.2: Chromosomal Location of Candidate Genes

Locus name	**Chromosomal location**
COL1A1	17q21.3-q22
COL1A2	7q21.3-22.1
COL2A1	12q13.1-q13.2
VDR	12q12-q14
PTHR1	3p22-p21.1
Calcitonin receptor	7q21.3
Calcium sensing receptor	3q13.3-q21
ER-α	6q25.1
Androgen receptor	Xq11.2-q12
IL-1	2q13
IL- 6	7p21-p15
IL-4	5q23-q31
IL-11	19q13.3-q13.4
TNF-α and -β	6p21.3
TGF-β	19q13.2
EGF	4q25
IGF-1	12q22-q23
CSF-1 (M-CSF)	1p21-p31
CSF-2 (GM-CSF)	5q23-q31.1
Osteopontin	4q11-q21
Osteocalcin	1q25-q31
Fibrillin	15q21.1
PTH	11p15.2-p15.1
PTHrP	12p12.1-p11.2

Candidates were chosen because of:

a) their role in normal bone structure and function (e.g. the calcitrophic hormones and their receptors);
b) murine models suggesting a role in control of BMD (e.g. osteoprotegerin knockout mice);
c) previous association studies in humans suggesting a role in determining BMD (e.g. Vitamin D receptor);
d) implication of a role in osteoporosis suggested by similar bone phenotype in other genetic disorders (e.g. COL1A1 mutations in Osteogenesis Imperfecta).

Obviously these categories are not mutually exclusive.

Many of the candidate genes in this study are discussed at length in Chapter 1 (Section 1.6 presenting their role in normal bone physiology and Sections 1.7 and 1.8 reviewing previous genetic studies of these candidate genes in osteoporosis). Therefore, to avoid repetition, the following section only covers aspects not discussed elsewhere.

Collagen type 1 alpha-1 and alpha-2

COLIA1 and COLIA2 code respectively for the two $\alpha 1$ and one $\alpha 2$ chains forming the triple helix of type 1 collagen present in skin, tendon and bone. Whilst genetically distinct, the two genes are similar to each other and to all the genes coding for collagen types 1, 2 and 3, in that they share an unusual and characteristic structure of a large number of relatively small exons (54 or 108 bp). Each exon begins coding for a glycine residue, and codes for a discrete number of gly-X-Y tripeptide units. COLIA1 is located

on chromosome 17 and consists of 51 exons with total size of 18 kb. COLIA2 on chromosome 7 consists of 52 exons over 35 kb (reviewed in http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?120150 and http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?120160).

Mutations of type 1 collagen result in Osteogenesis Imperfecta (OI), a disease characterised by brittle bones and multiple fractures (Sykes et al. 1990). OI has a wide range of phenotypic features, including differing degrees of bone fragility, blue sclerae, abnormal teeth, thin skin, weak tendons and hearing loss. The clinical heterogeneity of OI is due to the heterogeneity of type I collagen mutations. Around 200 different mutations have been described in OI patients, affecting both $\alpha 1$ and (more rarely) $\alpha 2$ chains, including substitutions, deletions, insertions, and formation of null alleles (the last manifesting as milder forms of OI). The most common defect in severe/lethal OI is a single base substitution causing replacement of a glycine residue for a bulkier amino acid, this impairing the tight helical structure of the triple helix (Kuivaniemi et al. 1997).

A fraction (1-3%) of patients with osteoporosis but lacking features of OI have been found to carry mutations of COL1A1 or COL1A2 (Spotila et al. 1994; Spotila et al. 1991).

A review of genetic studies in COL1A1, BMD and osteoporosis is presented in Chapter 1, with further discussion in Chapter 6.

Collagen type 2 alpha-1

COL2A1 codes for type 2 collagen, found in the cartilage and vitreous humour. COL2A1 is located on chromosome 12, with 75% sequence homology with COL1A1 and 63-67% homology with COL1A2 (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?120140). Mutations of COL2A1 result in chondrodysplasias, characterised by short-limbed dwarfism and skeletal deformities (Prockop et al. 1995). Mutations have also been reported to be a rare cause of early onset familial osteoarthritis (Ritvaniemi et al. 1995).

Vitamin D receptor

The VDR gene is located on chromosome 12, distal to COL2A1, and consists of eleven exons spanning over 75 kB. There are three non-coding exons at the start of the gene (exons 1A, 1B and 1C). Differential splicing of exons 1B and 1C results in three distinct mRNA isoforms. The hormone-binding domain of VDR is in the carboxy-terminal region (coded for by exons 7,8 and 9), as is the domain for heterodimerization with RXR. The DNA-binding domain is in the amino-terminal region, coded for by exons 2 and 3 with each exon coding separately for a zinc finger motif (reviewed in (Haussler et al. 1998)).

Mutations affecting VDR result in Vitamin D-dependent Rickets type II, otherwise known as hereditary resistance to $1,25(OH)_2D_3$. The disease is usually inherited in an autosomal recessive fashion. Affected children appear normal at birth but present in infancy with rickets and/or osteomalacia, no historical or biochemical evidence of

Vitamin D deficiency (in fact serum $1,25(OH)_2D_3$ levels may be extremely high), no historical or current evidence of calcium deficiency, hypocalcaemia, secondary hyperparathyroidism, and minimal response to administration of physiological doses of Vitamin D. In addition to effects upon calcitropic tissues, patients may also manifest alopecia and ectodermal anomalies including epidermal cysts and oligodontia.

Vitamin D-dependent Rickets type II is a clinically heterogeneous disorder, due to heterogeneity of cellular and molecular defects affecting VDR. These include: (a) defects of $1,25(OH)_2D_3$ binding, due to decreased receptor capacity (i.e. low number of binding sites) or decreased affinity of the receptor; (b) impaired nuclear localisation (including inability to heterodimerise with Retinoid-X receptor) or (c) decreased affinity of DNA binding (reviewed in (Haussler et al. 1998; Liberman et al. 1999) and (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?601769)). It is therefore not surprising that response to administration of high doses of $1,25(OH)_2D_3$ is highly variable. However, bone abnormalities can be substantially improved if not resolved by frequent intravenous overnight calcium infusions (al-Aqeel et al. 1993; Balsan et al. 1986). This suggests that VDR and $1,25(OH)_2D_3$ play a facilitative rather that obligate role in bone metabolism.

VDR homozygous knockout mutant mice have normal intrauterine and early postnatal growth and development prior to weaning. Subsequently, the mice fail to thrive, developing a syndrome very similar to human Vitamin D-dependent Rickets type II, with rickets, osteomalacia, hypocalcaemia, hypophosphataemia, secondary hyperparathyroidism and alopecia. Additionally the mice are infertile, with uterine

hypoplasia and impaired folliculogenesis secondary to impaired estrogen synthesis, and a proportion die in early life. Heterozygous VDR knockout mice appear phenotypically normal (Li et al. 1997; Yoshizawa et al. 1997). Dietary excess of calcium, lactose and phosphate may result in reversion to normal of many of the phenotypic abnormalities of VDR knockout mice, in particular bone mineralisation and PTH levels (Li et al. 1998).

The role of VDR polymorphisms in BMD determination has been studied at length and is reviewed in Chapter 1.

Parathyroid Hormone Receptor type 1

The gene for PTHR1 is at chromosome 3p22-21.1. The structure, physiology and regulation of PTHR1 are reviewed in Chapter 5.

Calcitonin receptor

The gene for the calcitonin receptor is on chromosome 7q21.3 (Nussenzveig et al. 1995). Like PTHR1, the calcitonin receptor is a G-protein coupled receptor with seven transmembrane domains. Several different mammalian calcitonin receptors exist and three distinct isoforms have been cloned from human tumour cell lines, due to alternative mRNA splicing (Gorn et al. 1992; Nussenzveig et al. 1995). The isoforms may have differential ligand specificity and/or responsiveness (Deftos et al. 1999). Calcitonin receptors are abundantly expressed on osteoblasts (Nicholson et al. 1986).

Calcium sensing receptor

The calcium sensing receptor is a member of the G-protein coupled receptor superfamily, with extracellular and intracellular domains and seven membrane-spanning domains. Binding of calcium ions to the extracellular domain triggers second messenger systems, resulting in suppression of PTH release in PTH cells, stimulation of calcitonin release in C-cells of the thyroid and decreased calcium reabsorption from renal tubular cells (reviewed in (Pearce et al. 1997)).

The calcium sensing receptor gene is located on chromosome 3q13.3-21. The gene consists of six exons and codes for a protein of 1078 AA (Garrett et al. 1995). Gene expression is widespread including many calcitropic tissues such as the parathyroid, thyroid C-cells, and renal tubular cells.

Inactivating mutations affecting the calcium sensing receptor gene result in hypercalcaemia. Heterozygosity results in autosomal dominant benign familial hypocalciuric hypercalcaemia. Homozygosity results in neonatal severe hyperparathyroidism (Pollak et al. 1993; Pollak et al. 1994). Activating mutations result in hypocalcaemia with low serum PTH and hypercalciuria. Vitamin D administration can precipitate nephrocalcinosis and acute renal failure (Pollak et al. 1994).

Estrogen Receptor-alpha

Two estrogen receptors are known in humans. The estrogen receptor-alpha (ER-α) gene on chromosome 6q25-27 spans 140kB and consists of eight exons with one known promoter region. The first exon codes for a regulatory domain, the DNA binding domain

contains two zinc fingers that are coded for by two separate exons, and the residual five exons make up the hormone-binding region (Ponglikitmongkol et al. 1988). The estrogen receptor-beta (ER-β) gene is on chromosome 14q22-24 also consisting of eight exons although spanning only 40 kB. High homology has been demonstrated between ER-α and -β in the DNA binding domain (96% conserved) and the ligand-binding domain (58%), however the regulatory domains of the two receptors are distinct. Alternative mRNA splicing results in several isoforms of each receptor each with different tissue expression (reviewed in http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?133430 and http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?601663). Variable ER-α subtypes in tumour cells expressing estrogen receptors (such as breast cancer cells) might result in differing responses to estrogen. Both ER-α and ER-β are expressed in human bone, present on osteoblasts, osteoclasts and osteocytes (Eriksen et al. 1988; Hoyland et al. 1997; Vidal et al. 1999).

ER-α knockout mice are completely infertile, lack breast tissue and have approximately 10% lower bone mass, supporting a facultative but not critical role of estrogen in skeletal development (Korach et al. 1996). ER-β knockout mice have reduced fertility in female but not male mice and normal breast tissue (Krege et al. 1998). The skeletal effects in ER-β knockout mice are yet to be reported.

Previous association studies of ER-α are discussed in Chapter 1.

Androgen receptor

The androgen receptor gene is on chromosome Xq11-12. It has a similar structure to the estrogen receptor genes, also consisting of eight exons with exon 1 coding for the modulatory domain, exons 2 and 3 the DNA-binding domain and exons 3 to 8 the hormone-binding domain. Multiple polymorphisms and mutations are known, with various mutations resulting in the androgen insensitivity syndrome, X-linked spinal and bulbar muscular atrophy, and prostate cancer (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?313700). Androgen receptors are also found on bone cells in similar concentrations to estrogen receptors (Colvard et al. 1989; Noble et al. 1999).

A modest study of LS BMD in pre- and perimenopausal women reported an association with an $(AGC)_n$ polymorphism in the androgen receptor gene (Sowers et al. 1999).

Interleukin-1

The genes coding for IL-1α, IL-1β and IL-1RA lie within a 1 cM region on chromosome 2 (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/maps.cgi?org=hum&chr=2).

The IL-1α gene consists of seven exons and is known to contain several polymorphisms. A 46 bp variable nucleotide repeat in intron 6 known to bind the transcription factor Sp1 has been reported to be associated with IL-1α production, with a trend for greater numbers of repeats to result in lesser gene expression (Bailly et al. 1996).

The IL-1β gene also consists of seven exons with several known polymorphisms (Langdahl et al. 2000). A single nucleotide polymorphism (C3954T) has been reported

to be associated with increased lipopolysaccharide-induced IL-1β production from peripheral blood monocytes (Pociot et al. 1992). A study of this SNP in patients with osteoporosis found no association of the polymorphism with osteoporotic fracture and BMD (Langdahl et al. 2000).

The IL-1RA gene comprises four exons, with two alternative first exons, coding for intracellular and secreted IL-1RA respectively. Several polymorphisms have been found in the coding region of IL1-RA in linkage disequilibrium with each other (Langdahl et al. 2000). Some but not all studies have also shown linkage disequilibrium extending as far as the IL-1β gene (Langdahl et al. 2000; Santtila et al. 1998). An association has been reported between an 86-bp VNTR in intron 2 and GM-CSF-stimulated monocyte production of IL-1RA (Danis et al. 1995).

Previous association studies of IL-1 are discussed in Chapter 1.

Interleukin-6

The gene for Interleukin-6 is located at chromosome 7p21. The osteoblast is the most prodigious source of IL-6 *in vivo* (Mundy 1999). Whilst its physiological role in the estrogen-replete state is uncertain, IL-6 and/or its receptor play critical roles in the changes in bone turnover induced by estrogen deficiency, with a similar role in androgen deficiency (see Chapter 1). In multiple myeloma, IL-6 is a powerful growth factor for myeloma cells and a major cause of the bone resorption in the disease (Klein et al. 1990). IL-6 has been studied in several association studies of BMD; these are discussed in Chapter 1.

Interleukin-4

The IL-4 gene is found on chromosome 5q31.1 in close proximity to IL-5, IL-13, fibroblast growth factors, and other haematopoietic growth factors (Sutherland et al. 1988).

Transgenic mice overexpressing IL-4 have markedly decreased bone formation, resulting in severe low-turnover osteoporosis (Lewis et al. 1993). Patients with hyper-IgE syndrome in which B-cells function as if exposed to excess IL-4 also develop low turnover osteoporosis (Leung et al. 1988).

Interleukin-11

The gene for IL-11 is located on chromosome 19q13.3-13.4 (McKinley et al. 1992). Interleukin-11 secretion by osteoblasts results in osteoclastogenesis and bone resorption (discussed in Chapter 1).

Tumour Necrosis Factors alpha and beta (TNF-α and TNF-β)

The genes for both TNF-α and TNF-β are closely linked and situated in the major histocompatibility complex, between HLA-B of class I and C2 of class III (Ragoussis et al. 1988). The two genes are encompassed in approximately 7 kB of DNA. Overall there is 30% homology between the genes. The last exons of the genes, however, which code for 80% of the secreted proteins, share greater (56%) homology (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?191160).

Transforming Growth Factor-β

The TGF-β1 gene consists of seven exons on 19q13.2, with exons 5,6 and 7 coding for active TGF-β1. Several polymorphisms of TGF-β1 have been studied in association studies in osteoporosis (see Chapter 1).

TGF-β1 homozygote knockout mice have significantly lower total bone mineral content and growth, although BMD is unchanged (Geiser et al. 1998). Mice lacking an important extracellular matrix proteoglycan, biglycan, which binds TGF-β and negatively regulates its function, develop osteoporosis and exhibit reduced growth rate (Xu et al. 1998). In contrast to the effects of TGF-β1, TGF-β2 overexpression in transgenic mice causes osteopaenia due to increased bone remodelling (Erlebacher et al. 1998).

Epidermal Growth Factor (EGF)

EGF is reviewed in Chapter 1.

Colony Stimulating Factor –1 and –2

The roles of CSF-1 and -2 in bone are reviewed in Chapter 1.

In mice, a point mutation in the coding region of CSF-1 results in murine osteopetrosis (Yoshida et al. 1990). The mice have impaired osteoclastogenesis with osteoclasts absent from trabecular surfaces. Administration of recombinant CSF-1 results in restoration of osteoclastogenesis, bone resorption and marrow cavity formation (Felix et al. 1990; Kodama et al. 1991). In humans however osteopetrosis is a heterogeneous disorder and the pathological bases of the many types of osteopetrosis are not known.

Osteopontin

Osteopontin is present abundantly in the extracellular matrix of bone and has an important role in bone remodelling (reviewed in Chapter 1). The osteopontin gene is on chromosome 4q21-23 and, in common with other bone glycoproteins, contains the coding sequence for the consensus motif RGD (Arg-Gly-Asn) for cell adhesion. Osteopontin gene expression in bone is regulated by $1,25(OH)_2D_3$ through a vitamin D response element (Staal et al. 1996). Osteopontin gene expression is also induced in response to mechanical stimulation (Terai et al. 1999).

Osteopontin knockout mice were reported not to have skeletal abnormalities but rather altered wound healing with abnormal collagen fibrillogenesis and disorganized matrix production (Liaw et al. 1998). However, osteopontin knockout mice are resistant to bone resorption induced by ovariectomy and by PTH, despite increased numbers of osteoclasts (Yoshitake et al. 1999).

Osteocalcin

The osteocalcin gene contains four exons and is located on chromosome 1. The osteocalcin gene is thought to arise from a common ancestral sequence with coagulation factor IX. There is high sequence homology between them, particularly the residues involved in post-translational vitamin K-dependent γ carboxylation (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?112260). Osteocalcin gene expression is regulated by a vitamin D response element in its promoter region (Kerner et al. 1989).

Osteocalcin homozygous knockout mice have increased bone mass due to increased bone formation, without abnormalities of mineralisation or bone resorption (Ducy et al. 1996).

Fibrillin

The fibrillin gene is quite large (110kB) with a very fragmented coding region (65 exons). Fibrillin contains multiple (46) EGF-like domains, most of which contain a consensus sequence capable of binding calcium. The EGF-like domains form a helical structure stabilized by calcium. Mutations affecting calcium binding affect the stability of this helix and hence the microfibril structure (Handford et al. 1995). Most of the EGF-like domains are coded individually by a discrete exon hence the large size of the gene (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?134797).

Fibrillin is found in many other connective tissues in addition to bone. Mutations of fibrillin are known to cause the Marfan's syndrome, a disease of connective tissue characterised by ocular, skeletal and cardiovascular abnormalities including ectopia lentis, long bone overgrowth, arachnodactyly, scoliosis, aortic root dilatation, mitral valve prolapse, aortic aneurysm, and dural ectasia (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?154700). Low bone mineral density has been described in both men and women with the Marfan's syndrome (Carter et al. 2000).

Parathyroid Hormone

The gene for PTH is located on chromosome 11p15.3-p15.1, consisting of three exons coding respectively for the 5' UTR, a 25AA signal peptide, and PTH with the 3' UTR (Kemper 1986). Translation of PTH mRNA initially produces preproPTH (115 AA).

The signal peptide is then cleaved to form proPTH (90 AA), before further cleavage of a hexopeptide results in the functional hormone. In all mammalian species investigated, PTH is a single-chain polypeptide of 84 AA. The amino-terminal region demonstrates high homology at in all vertebrates examined (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?168450).

PTH expression is regulated by $1,25(OH)_2D_3$ through a vitamin D response element. Calcium and phosphate also regulate PTH gene expression, through post-transcriptional protein-RNA interactions at the PTH mRNA 3'UTR (Silver et al. 1999).

Mutations of the PTH gene have been found in some autosomally inherited forms of hypoparathryoidism (Arnold et al. 1990; Bilous et al. 1992; Parkinson et al. 1992; Sunthornthepvarakul et al. 1999). Non-coding polymorphisms of the PTH gene have also been reported (Mullersman et al. 1992).

Parathyroid Hormone-related Peptide

The gene for PTHrP is located on chromosome 12p12.1-11.2. It consists of three promoter regions, with six coding exons. The similarity in structure between the PTHrP and PTH genes and PTH and PTHrP imply a common ancestral gene (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?168470). A complex pattern of alternative splicing results in several mRNA species and three common protein isoforms of 139, 141 and 173 AA in length, which may have differing biological activity.

No polymorphisms of this gene have been reported to date.

4.2.3 Candidate Gene Genotyping

Microsatellite markers located close to (≤5cM from physically mapped loci, or spanning the interval containing the gene for genes of imprecise location) or within the candidate genes were identified from the Genome Database 5.6 and 6.0 (http://www.hgmp.mrc.ac.uk/gdb/), Whitehead Institute STS database (http://www-genome.wi.mit.edu), the Medical Research Council (UK) microsatellite set (Reed et al. 1994), and CEPH/genethon maps (http://www.cephb.fr/ceph-genethon-map.html). The markers selected for each candidate gene are presented in Table 4.2.2. Their genetic location is presented in Appendix 4.3.

Table 4.2.2: Microsatellite markers amplified for candidate genes

Candidate gene	Microsatellite Marker
Androgen receptor	DXS1275, DXS986
COL1A1	D17S791, D17S1604, D17S807, D17S789
COL1A2 and calcitonin receptor	D7S2431, COL1A2 intragenic dinucleotide
COL2A1 and VDR	D12S368, D12S1586, D12S1702, D12S83
CSF-1	D1S290, D1S198, D1S216, D1S207
Calcium sensing receptor	D3S1309, D3S1593, D3S1279, D3S1268
EGF	D4S1572, D4S406, D4S193, D4S430, D4S429, D4S247
ER-α	D6S1654, D6S441, D6S1577, ER intragenic dinucleotide
Fibrillin	MTS1, MTS4
IGF-1	D12S78, D12S79, D12S86, IGF-1 intragenic dinucleotide
IL-1	D2S160, D2S2265, IL1α intragenic dinucleotide
IL-4 and CSF-2	D5S2057, D5S393, D5S2017, D5S178
IL-6	D7S503, D7S493, D7S673
IL-11	D19S412, D19S866
Osteocalcin	D1S2815, D1S238
Osteopontin	D4S392, D4S3042, D4S395
PTH	D11S902, D11S1755, D11S915
PTHrP	D12S364, D12S1699
PTHR1	D3S3559, D3S1289
TGF-β	D19S422
TNF	TNF alpha intragenic dinucleotide, D6S276

Microsatellite markers were synthesized by Sigma-Genosys Ltd., Pampisford, Cambridgeshire, and by the Medical Research Council (UK) (Reed et al. 1994).

Markers were amplified and genotyped as described in Chapter 2.

4.2.4 Statistical Analysis

Allele numbers and frequencies were calculated from the observed data. Physical mapping data were obtained from the Whitehead Institute STS database (http://www-genome.wi.mit.edu), Genome Database 6.0 (http://www.hgmp.mrc.ac.uk/gdb/) and the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Recombination mapping using the programme LINKMAP (Lathrop et al. 1984) was used to determine the location of microsatellites that had not previously been physically mapped relative to those that had been.

The original 115 families were divided into 165 nuclear units containing at least one sibling pair for analysis, using the programme 'GAS' (A. Young, unpublished). BMD was expressed as z-scores (adjusted for age and gender). BMD was also adjusted for effects of body size by regressing BMD against BMI.

Quantitative trait analysis was performed using the statistical programmes MapMaker/Sibs (Kruglyak et al. 1995) and ACT (Amos et al. 1996). MapMaker/Sibs was used to perform Haseman-Elston (Haseman et al. 1972) and variance components analyses, although in all cases the variance components analysis was more significant than the corresponding Haseman-Elston analysis.

When more than one pair of siblings can be drawn from each sibship, statistical bias may be introduced due to the non-independence of siblings (Hodge 1984). Whilst MapMaker/Sibs contains various settings (e.g. 'all pairs') to adjust for this bias, the correction is acknowledged to be conservative such that whilst type 1 error is not

increased, type 2 error is (i.e. power is decreased) (Davis et al. 1997). Single results presented below are for the 'all pairs' setting with correction for this bias. Where a range of LOD scores is presented, the lower value is for the 'all pairs' setting with correction, and the higher value for no correction. The true LOD score lies between the two. For all markers achieving a LOD score $\geq$1.0 using MapMaker/Sibs with the 'all pairs' setting, variance components analysis was also performed using ACT. As described in Chapter 1, variance components modelling models the covariances between family members and no correction for multiple sibling pairs per family is required (Amos et al. 1996). Theoretic and simulation studies have also suggested that variance components analyses such as ACT which make joint use of all sibship data are more powerful than analyses which are restricted to pairwise comparisons such as MapMaker/Sibs (Amos et al. 1997; Williams et al. 1999; Wright 1997).

Correction for ascertainment was performed using ACT to adjust for the stringent ascertainment scheme used to recruit families through a proband with an extreme BMD value.

Prior information about the mean population BMD z-score (i.e. zero) was used in the analysis using ACT, potentially increasing its power. Fixing the mean BMD to zero is valid if the reference population is representative of that from which the study population was drawn. Whilst the reference population and British population are likely to be similar, this has only formally been demonstrated at LS (Ryan et al. 1993), and therefore analysis was performed both fixing the mean z-score and estimating it directly from the study population.

MapMaker/Sibs was used perform multipoint analysis, which was not available using ACT.

For ACT, significance values were converted from log-likelihood ratios to χ^2 statistic and thence to LOD scores using the formula LOD = χ^2 /2 ln 10.

4.3 Results

Fifteen markers from eight candidate regions achieved LOD scores ≥1.0 by MapMaker/Sibs (see Table 4.3.1). Results for MapMaker/Sibs are all for variance components analysis as in all cases this was more significant than the corresponding Haseman-Elston analysis. The strongest linkage achieved was marker D3S1289 (FN LOD score 2.7-3.5, depending on correction for number of siblings per family). Marker D3S3559 also showed moderate evidence of linkage to both FN and LS BMD with LOD scores 1.5-2.2 and 1.3-1.6 respectively.

Of the fifteen markers achieving a LOD score of ≥1.0 using MapMaker/Sibs, six markers from three regions achieved a LOD score ≥1.0 with the ACT programme (D2S160, D3S1289, D3S3559, D7S503, D12S1586, D12S83). The ACT variance components analysis is given in Table 4.3.2. Using ACT, very little difference was observed between values where the population mean was fixed and where the mean was estimated from the study population.

The single and multipoint linkage results for all the candidate genes are presented in Appendix 4.3.

Table 4.3.1:

MapMaker/Sibs Variance Components Results for All Markers with LOD Score ≥1.0

Results are presented using conservative correction for number of siblings per family.

		FN Results		LS Results	
Candidate Gene	**Marker**	**Two point**	**Multipoint**	**Two point**	**Multipoint**
IL-1	D2S160	0.5	1.4*	0.7	0.4
PTHR1	D3S3559	1.5*	1.8*	1.3*	0.8
PTHR1	D3S1289	2.7*	2.0*	0.3	0.4
EGF	D4S430	1.3*	1.4*	0.4	0.3
EGF	D4S429	1.8*	1.6*	0.2	0.3
EGF	D4S427	0	1.4*	0	0.3
IL-4	D5S2057	1.1*	0.3	0.0	0.0
IL-4	D5S2017	1.2*	0.9	0.3	0.2
IL-4	D5S178	0.1	1.1*	0.0	0.2
ER-α	D6S1577	0.1	0.4	0.5	1.4*
IL-6	D7S503	0.6	0.3	1.2*	1.1*
COL2A1/VDR	D12S1586	1.0*	0.4	0.7	0.8
COL2A1/VDR	D12S83	0.0	0.3	1.7*	1.3*
COL2A1/VDR	D12S1702	0.9	0.4	0.1	1.1*
COL1A1	D17S807	1.7*	0.6	0.5	0.3

*LOD score ≥1.0

Table 4.3.2: Results of Variance Components Analysis using the Programme ACT

Results are presented with the mean BMD z-score both estimated from the study population, and with the mean fixed at the mean population z-score (zero).

			FN results		LS results	
Candidate Gene	**Marker**	**Mean z-score**	**LOD score**	**p-value**	**LOD score**	**p-value**
IL-1	D2S160	Estimated	1.0	0.03*	0.5	0.1
		Fixed	0.8	0.05*	0.4	0.2
PTHR1	D3S3559	Estimated	1.4	0.01*	1.1	0.02*
		Fixed	1.6	0.005*	1.3	0.01*
PTHR1	D3S1289	Estimated	2.8	0.0003*	0.5	0.1
		Fixed	3.2	0.0001*	0.4	0.2
EGF	D4S430	Estimated	0.2	0.3	0.1	0.5
		Fixed	0.2	0.3	0.1	0.5
EGF	D4S429	Estimated	0.1	0.5	0.1	0.5
		Fixed	0.1	0.5	0.1	0.5
EGF	D4S247	Estimated	0.3	0.3	0.1	0.5
		Fixed	0.2	0.3	0.1	0.5
IL-4	D5S2057	Estimated	0.1	0.4	0.1	0.5
		Fixed	0.2	0.4	0.1	0.5
IL-4	D5S2017	Estimated	0.6	0.09	0.4	0.2
		Fixed	0.7	0.06	0.3	0.2
IL-4	D5S178	Estimated	0.4	0.2	0.1	0.5
		Fixed	0.5	0.1	0.1	0.5
ER-α	D6S1577	Estimated	0.1	0.5	0.2	0.4
		Fixed	0.1	0.5	0.2	0.3
IL-6	D7S503	Estimated	0.2	0.3	1.3	0.01*
		Fixed	0.3	0.2	1.4	0.009*
COL2A1/ VDR	D12S1586	Estimated	0.3	0.2	1.2	0.02*
		Fixed	0.3	0.2	1.3	0.01*
COL2A1/ VDR	D12S83	Estimated	0.1	0.4	1.4	0.009*
		Fixed	0.6	0.08	1.4	0.009*
COL2A1/ VDR	D12S1702	Estimated	0.1	0.5	0.1	0.5
		Fixed	0.1	0.5	0.1	0.5
COL1A1	D17S807	Estimated	0.3	0.3	0.1	0.5
		Fixed	0.3	0.2	0.1	0.5

* p-value ≤ 0.05

4.4 Discussion

This study provides evidence for a role of polymorphisms of several genes in the aetiology of osteoporosis. 'Suggestive' evidence of linkage was observed between markers lying in the region of the PTHR1 gene and BMD. Seven other loci (IL1, EGF, IL-4, ESR1, IL-6, COL2A1/VDR, COL1A1) showed some evidence of linkage, although none of these linkages were strong. The results are consistent with previous segregation studies of BMD indicating the trait most closely fits a polygenic model of inheritance (Gueguen et al. 1995).

Quantitative trait linkage analysis is more powerful than dichotomising a trait into a qualitative phenotype. Therefore linkage analysis was performed using BMD as the phenotype rather than the clinical entity of osteoporosis. Correction of BMD for bone size was performed in order to approximate a calculated volumetric bone density (as discussed in Chapters 1 and 3). Using BMI-adjusted BMD also allowed analysis of the genetic factors determining BMD independently of those determining body size. BMI has previously been shown to contribute up to 15% of the overall heritability of BMD (Sowers et al. 1992), accounting for a larger proportion of overall BMD variance than for height, fat mass or weight (Jouanny et al. 1995). BMI is a strong predictor of BMD (as discussed in Chapter 1). The genes determining BMD may have a pleiotrophic effect and contribute to the heritability of body size as well as BMD (Nordstrum et al. 1999). In this circumstance, adjusting for BMI would result in loss of power to detect such loci. However, an analysis of heritability of fat mass, lean mass and BMD found that the heritability of each was due to specific genetic factors (Nguyen et al. 1998). Genetic

factors affecting lean body mass had a non-significant influence on BMD heritability (accounting for <15%). Genetic factors affecting fat mass also had a non-significant influence upon BMD heritability at LS or FN although a small genetic correlation was noted with total BMD ($r = 0.31$). However, significant environmental correlations were noted between both fat and lean mass and BMD; thus the correlations between either fat or lean mass and BMD were due to shared environment, not shared heritability. It should be mentioned however that the twin zygosity of this work was established by questionnaire, which was subsequently shown to be inaccurate in some cases; these results therefore are suspect. Nonetheless, adjusting for BMI will enhance the power to detect linkage with BMD by decreasing the variance of BMD due to environmental effects.

As discussed in Chapter 3, ascertainment schemes where probands are selected for extreme quantitative trait values are more powerful for detection of quantitative trait loci than randomly recruited families. For this study families were recruited through a single affected proband (BMD z-score <-2.0; corresponding to the lowest 2.275% of the age- and sex-matched population distribution, in addition to having osteoporosis as defined by WHO (Kanis et al. 1994)). Such stringent ascertainment schemes may introduce bias into the linkage analysis; however simulation studies using MapMaker/Sibs and other programmes suggest that this bias is likely to be trivial (Iyengar et al. 1997; Slager et al. 1997). Further, failure to correct for ascertainment bias tends to lead to loss of power, rather than to inflation of type 1 error (Fisher et al. 1999; Marlow 2001) (see Chapter 3 for the effects on heritability estimates using variance components analysis with and

without adjusting for ascertainment bias). Nonetheless, linkage analysis using ACT was adjusted for ascertainment bias.

The appropriate level of correction to apply when testing multiple loci for linkage in candidate gene screening is controversial. An extreme position is that whole-genome thresholds should also be applied to candidate gene screens, on the presumption that an infinitely dense whole-genome screen (arguably the gold standard for linkage analysis) will eventually be performed (Lander et al. 1995). Lander and Kruglyak suggested the terms 'suggestive linkage' for a p-value $\leq 7.4 \times 10^{-4}$ corresponding to a LOD score of 2.2, statistical evidence that would be expected to occur once by chance in such a dense genome screen; and 'significant linkage', a p-value $\leq 2.2 \times 10^{-5}$ and LOD score of 3.6, statistical evidence that would be expected to occur once by chance in twenty such infinitely dense genome screens. There is however disagreement amongst statistical geneticists regarding the principle of applying thresholds and the accuracy of the thresholds set (Curtis 1996; Witte et al. 1996). A simulation study using data derived from a real whole genome screen suggested that a LOD score of 3.2 is equivalent to a whole genome p-value of 0.05 (the probability below which one or fewer false positives would be expected to occur in 20 whole genome screens) (Sawcer et al. 1997). An alternative view of thresholds for candidate gene studies is that Bonferroni correction should be applied for the number of independent loci investigated, whereby the p-value obtained is multiplied by the number of independent observations drawn from the same data set to give the final significance value.

By either standard the results obtained at femoral neck with the markers D3S1289 and D3S3559, both with two point and with multipoint analysis, are striking. They fulfil the criteria for suggestive linkage as above, or, if Bonferroni correction is used, the corrected p-value for linkage between marker D3S1289 and BMD is 0.002, a highly significant result.

The markers D3S1289 and D3S3559 lie close to the candidate gene PTHR1 (D3S1289, PTHR1 and D3S3559 lie at 62.7cM, 65.1-67.7cM and 69.1cM from the p-telomere of chromosome 3 respectively). As discussed above, linkage studies cannot pinpoint genes of interest for quantitative trait loci and further mapping and functional work are needed to establish that it is PTHR1 rather than another gene in linkage disequilibrium with these markers that is responsible for the observed linkage with BMD. Nonetheless, PTHR1 was chosen as a candidate gene because of its known role in bone physiology (reviewed in Chapter 5). Mutations resulting in constitutively activated PTHR1 cause Jansen-type metaphyseal chondrodysplasia, a form of short-limbed dwarfism associated with hypercalcaemia due to increased bone resorption, hypophosphataemia, and normal to high PTH levels (Kruse et al. 1993; Schipani et al. 1996). Although abnormalities of endochondral ossification are the principle phenotypic abnormality in this condition, increased bone resorption without sufficient compensatory bone formation does occur (Kruse et al. 1993). In transgenic mice, targeted expression of constitutively active human PTH/PTHrP receptors in growth plate chondrocytes causes delayed endochondral bone maturation and reduced bone mineralization at birth. These effects are due to decelerated chondrocyte maturation but also due to inhibition of vascularisation, a key step in ossification. The histological abnormalities resolve by two weeks of age,

probably because of decreasing numbers of cells expressing the transgene (Schipani et al. 1997). The influence of constitutive PTH receptor activation in osteoblasts has not been similarly assessed, but would be anticipated to cause osteolysis and osteopaenia, as occurs in chronic PTH excess. Mutations in the PTHR1 gene resulting in loss of function of the PTHR1 receptor have been identified in patients with Blomstrand's chondrodysplasia, which is characterised by advanced endochondral bone maturation and increased bone density (Jobert et al. 1998; Zhang et al. 1998) and homozygous knockout mice have a similar phenotype. Both Blomstrand's and Jansen's chondrodysplasias result from mutations of PTHR1 and result in a severe skeletal phenotype. More subtle polymorphisms of PTHR1 inducing milder degrees of receptor activation or suppression may underlie population variance in BMD. This is discussed further in Chapter 5.

Other sites with evidence of linkage with BMD included COL1A1, IL-1, IL-6, ER-α and COL2A1/VDR, all of which have previously been studied in association studies and are reviewed in detail in Chapter 1. Linkage was also observed with IL-4 and EGF, which are novel areas of interest.

Of those markers achieving a LOD score of $\geq$1.0 using the variance components part of MapMaker/Sibs, only six markers (D2S160, D3S1289, D3S3559, D7S503, D12S1586, D12S83) from three other regions (IL-1, IL-6 and PTHR1) achieved a LOD score $\geq$1.0 with ACT (also variance components analysis). The more accurate of the two programmes cannot be known until specific comparisons between them are made. However, the use of cross-generational comparisons of BMD in ACT may be

inappropriate due to differing proportions of BMD from ascertainment of peak bone mass and subsequent bone loss in differing generations of a family (discussed further below).

There is strong evidence from association studies of involvement of COL1A1 in determining BMD (see Chapter 6). Whilst linkage was observed at this locus using MapMaker/Sibs, the level of significance was not high. As mentioned before, genetic studies in these families may be biased towards heritability of peak bone mass rather than bone loss, and this weak result may reflect the relative importance of COL1A1 in determining each component of total BMD.

Both COL2A1 and VDR are located on chromosome 12, separated by less than 740 kB (Pedeutour et al. 1994). Uitterlinden and colleagues recently reported distinct associations of VDR and COL2A1 with separate features of osteoarthritis (Uitterlinden et al. 2000). In contrast to association work, the two genes cannot be examined independently in linkage studies due to their close genetic relationship. The area of maximum multipoint analysis was proximal rather than distal, which might suggest linkage more to COL2A1 rather than VDR. However, as discussed in Chapter 5, the shape and maximal height of multipoint linkage curves can be very misleading relative to the actual location of the disease-causing gene.

As an extension of this point, it must be recognised that although markers were chosen close to or within candidate genes, it cannot be stated with certainty that the linkage observed was due to the intended genes. For example, the gene for fibroblast growth factor, a cytokine resulting in bone cell formation and osteoblast functioning, is

sufficiently genetically close to IL-4 for effects at either gene to be indistinguishable by linkage analysis.

The intricate interrelations between bone cytokines and gonadal hormones, particularly in mediating the bone loss associated with menopause (reviewed in Chapter 1), makes the linkage results at candidates IL-1, IL-6 and ER-α fascinating. The synergistic and cascade-like pathways involved mean that subtle polymorphisms in any of the cytokines could have profound effects upon the final skeletal phenotype. To dissect out the relative importance of each cytokine will be difficult. In qualitative diseases, linkage analysis can be performed conditional upon a previously identified locus (Cordell et al. 1995). This cannot yet be done for quantitative diseases (M. Farrell, personal communication).

The level of significance of linkage observed at the PTHR1 locus was greater at FN than at LS, although there was at least moderate linkage at both sites. BMD at LS is affected by many artefacts, which would reduce the power of linkage analysis to detect genes purely affecting BMD at this site. Alternatively, this may reflect genetic heterogeneity in the causation of osteoporosis at these two sites, consistent with epidemiological evidence of site specificity of BMD heritability (discussed in Chapter 3). Differences in linkage results between FN and LS were also seen at the IL-1 locus (greater at FN) and IL-6 and VDR/COL2A1 loci (both greater at LS), using both MapMaker/Sibs and ACT programmes.

In addition to site-specificity of genetic effects, environmental variables (such as calcium intake) may interact with genetic influences in a site-specific manner (discussed in Chapters 1 and 3). Environmental influences may differ substantially between

populations, and linkage analysis of these candidate genes in different populations may reveal genetic effects not detectable in this population.

The results presented in Chapter 3 also suggest that there may be gender effects upon BMD heritability at different skeletal sites. The mixed sex study design employed would mask any such gender-specific genetic effects. Further, this linkage analysis cannot differentiate between effects due to genes controlling peak bone mass or rate of bone loss. Therefore this study should not be seen as excluding an effect of any of the genes studied on BMD in particular situations.

Previous full genome screens have not demonstrated linkage to the areas identified in this study. This may be due to ethnic heterogeneity of BMD heritability. The families in this study were British Caucasians, in contrast with previous linkage studies in French Canadians, Greeks, and Ashkenazi Jews (Devoto et al. 1998; Spotila et al. 1996), Caucasian- and Afro-Americans (Koller et al. 1998) and Chinese (Niu et al. 1999). Certainly these studies lacked sufficient power to exclude any of the candidate areas examined here – and in most cases were insufficiently powered to detect the moderate genetic effects reported in this study (Gu et al. 1997). Similarly, the power of this study is at best moderate, and genes contributing only a small proportion of total heritability of BMD may not have been detected. Greater power will require investigation of more families and utilisation of non-parametric linkage analysis packages capable of multipoint analysis of general (rather than nuclear) pedigrees.

In conclusion, this candidate gene linkage study has demonstrated 'suggestive' linkage of the PTHR1 locus with BMD, with lesser evidence of a role for the genes COL1A1,

COL2A1/VDR, EGF, ESR1, IL-1, IL-4 and IL-6. The strength of linkage of PTHR1 and BMD, and the prior evidence of an osteoporosis-associated phenotype with PTHR1 mutations (Schipani et al. 1996), make this an important candidate for further study.

4.5 Further Candidate Gene Linkage Study – Introduction

The OPG/RANKL/RANK axis of bone cell signalling was first reported after the above candidate gene study was performed. These three proteins appear pivotal in control of osteoclastogenesis (reviewed in Section 1.6.4.1) and thus were obvious additional candidate genes of interest.

The gene for OPG is located on chromosome 8q23-24 (Morinaga et al. 1998) and consists of one promoter region and five exons (Langdahl et al. 2000). 12 polymorphisms have been reported within the OPG gene. Two polymorphisms in the promoter region and one in exon 1 (resulting in a AA change) have been found to be associated with fracture and low BMD at LS (for all three) and femoral neck (for one promoter polymorphism), with a suggestion of an osteoporotic haplotype associated with fracture and low BMD at LS (Langdahl et al. 2000).

The gene for RANKL is at chromosome 13q14. No polymorphisms of the RANKL gene have been reported.

The RANK gene is located on chromosome 18q22.1(Anderson et al. 1997). Several polymorphisms and mutations of the RANK gene have been described. Linkage of this

gene with familial expansile osteolysis has recently been demonstrated (Hughes et al. 2000). Mutations affecting exon 1 were found to affect expression levels and prevent normal cleavage of the signal peptide, thought to result in higher intracellular levels of RANK translation products and increased signal transduction.

Further, two genome-wide linkage studies were published, with some evidence of linkage of BMD with chromosome 11q (Devoto et al. 1998; Koller et al. 1998). Maximal linkage was reported with microsatellite marker cd3d in the first study (Devoto et al. 1998).

Therefore a further candidate gene study of OPG, RANKL, RANK and marker cd3d was undertaken to examine linkage of these loci with BMD.

The analyses in the candidate gene study presented above required complex family pedigrees to be split into nuclear families, with subsequent loss of power. Statistical packages capable of analysing extended pedigrees (e.g. Sequential Oligogenic Linkage Analysis Routines (SOLAR) (Almasy et al. 1998)) should have greater power to detect linkage. Therefore part of the aim of this additional linkage work was to compare the results from a statistical programme that analysed only nuclear families with one capable of using information from more complex pedigrees.

4.6 Methods

4.6.1 Family Members

The same families were used as for the above study.

4.6.2 Candidate Gene Identification and Genotyping

Microsatellite markers located close to the three candidate genes were identified from the Genome Database 6.0 (http://www.hgmp.mrc.ac.uk/gdb/) and the Whitehead Institute STS database (http://www-genome.wi.mit.edu). The markers selected for each candidate gene are presented in Table 4.6.2 with genetic distances in Kosambi cM.

Table 4.6.2: Microsatellite markers amplified for candidate genes OPG, RANKL, RANK and chromosome 11q

Candidate area	**Microsatellite marker**	**Position in Kosambi cM**
RANKL	d13s218	35.3
RANKL	d13s263	40.4
RANKL	d13s153	47.5
RANK	d18s64	83
RANK	d18s1147	88.8
RANK	d18s68	94.4
RANK	d18s465	98.9
OPG	d8s1784	116.8
OPG	d8s1008	123.5
OPG	d8s514	128.9
OPG	d8s1793	135.5
OPG	d8s284	142.7
OPG	d8s256	147.6
OPG	d8s1837	155.5
OPG	d8s1717	162.8
11q	CD3D	113.9-117

Microsatellite markers were synthesized by Gibco/Life Technologies^{TM} (Paisley, UK). Markers were amplified and genotyped as described in Chapter 2.

4.6.3 Statistical Analysis

Allele numbers and frequencies were calculated from the observed data. Physical mapping data were obtained from the Whitehead Institute STS database (http://www-genome.wi.mit.edu).

Linkage was performed using BMD expressed as z-scores (adjusted for age and gender). BMD was also adjusted for effects of body size by regressing BMD against BMI.

Linkage analysis using nuclear families and the programme MapMaker/Sibs was carried out as described above, with both two-point and multipoint linkage analysis. Both Haseman-Elston and variance components analyses were performed with and without adjustment for number of siblings drawn from each family, and therefore linkage results from MapMaker/Sibs are presented as a range.

General pedigree nonparametric quantitative linkage analysis was performed using SOLAR. SOLAR uses variance components modelling to analyse linkage. Heritability estimates were obtained by within-programme variance components analysis, correcting for ascertainment bias.

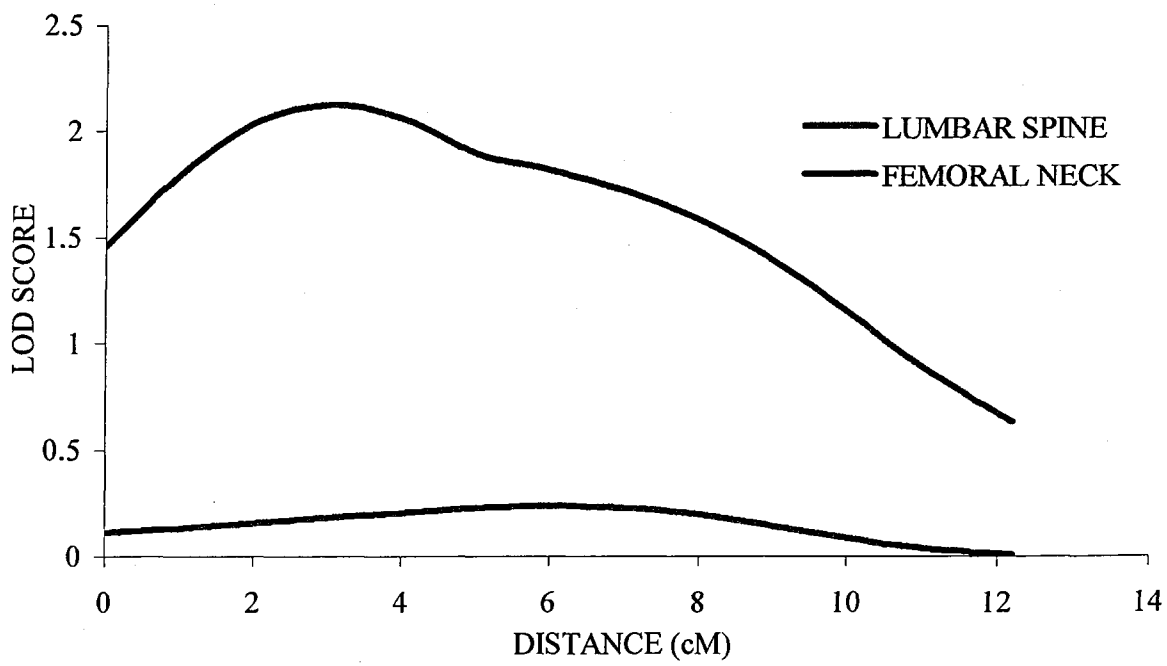
4.7 Results

4.7.1 Results using MapMaker/Sibs

Suggestive evidence of linkage of BMD at LS with RANKL was found, with maximum LOD scores (MLS) of 1.7-2.1 using variance component multipoint analysis. Maximum linkage with BMD was seen at markers d13s218 and d13s263 using variance components analysis. There was no evidence of linkage at FN (MLS 0.37).

No evidence of linkage with OPG, RANK, or 11q was observed.

Diagram 4.7.1: Multipoint Linkage Analysis of RANKL with BMD



4.7.2 Results using SOLAR

Multipoint linkage analysis of these families using SOLAR was unsuccessful. However, two-point analysis was available for markers d13s218 and d13s263. These results are presented in Table 4.7 with the two-point results for these markers obtained using MapMaker/Sibs.

Table 4.7: Two-point Linkage Results using MapMaker/Sibs and SOLAR

	Mapmaker/Sibs		**SOLAR**	
Microsatellite marker	**LS**	**FN**	**LS**	**FN**
d13s218	0.43-0.49	0.24-0.42	0.39	0.64
d13s263	0.49-0.52	0.00-0.08	0.18	0.22

4.8 Discussion

This further candidate gene work demonstrated evidence of linkage of LS BMD with RANKL but not OPG or RANK. No linkage was observed with the microsatellite marker cd3d on chromosome 11q. No evidence of linkage of any locus with FN BMD was demonstrated. However, as for the previous candidate gene study, there is insufficient power to exclude linkage of these loci with BMD.

The computational time required for establishing IBD sharing using SOLAR was prohibitive. Attempts to run the analysis as a whole were ineffective, with each microsatellite marker requiring over a week for analysis, and computer instablity

resulting in repeated loss of linkage data. To circumvent loss of all the linkage results each time the computer systems required rebooting, the data was split into individual markers and analysed separately. Despite this, only two microsatellites were successfully analysed, taking a fortnight of computation. Only two-point analysis was performed. Alternative packages capable of analysing general pedigrees (e.g. Genehunter (Kruglyak et al. 1996)) were also considered. However, Genehunter can only model vector-descent pathways in families with a maximum of 8 founding members. Attempts to run this programme with the complex pedgrees of these families resulted in the programme discarding multiple individuals from the pedigrees, causing considerable loss of power. Further, Genehunter has no capcity for ascertainment correction.

The two-point linkage results from SOLAR are similar to the two-point data obtained using MapMaker/Sibs. Thus it is arguable whether the considerably greater effort to analyse complex pedigrees did in fact result in improved power to detect linkage. No comparison of the multipoint analyses was made, however.

Further, the biological validity of comparing BMD of relatives of a complex pedigree is not certain. At any one point in time, bone mineral density consists of both peak bone mass obtained and subsequent bone loss. The relative contributions of these two factors to total BMD are likely to be most similar amongst siblings who are of comparable age, than amongst relatives separated by generations. For example, the proportion of total BMD due to bone loss will be much greater in postmenopausal mothers than in premenopausal children. Including menopausal status as a covariate would diminish, but

not remove, this bias. Therefore, linkage analysis of BMD in complex pedigrees may involve comparison of different phenotypes.

This would be consistent with the evidence of previous genetic epidemiological studies, where heritability estimates obtained from family studies have in general been considerably lower than those obtained using siblings (presented in Section 1.4.2).

In summary, linkage of BMD at LS was demonstrated with RANKL. Linkage analysis of BMD utilising the complex pedigrees available (rather than dividing them into nuclear families) did not result in improved linkage detection. This may have been due to potentially avoidable computational limitations. However, it may also be due to differing contributions to the overall phenotype of BMD amongst relatives of different generations.

Chapter 5: The Parathyroid Hormone Receptor type 1: High Density Linkage Mapping and Mutation Screening

5.1 Introduction

The candidate gene linkage study presented in Chapter 4 identified linkage of BMD with markers d3s1289 and d3s3559, located at 62.7cM and 69.1cM respectively from the p-telomere on chromosome 3. The maximum FN LOD scores obtained were 2.7-3.5 with d3s1289 and 1.5-2.2 with d3s3559. The maximum LS LOD scores were 1.3-1.6 with d3s3559. The PTHR1 locus is located at 65.1-67.7cM. Although this linkage result could be due to the effects of any of the genes in the same chromosomal area, the PTHR1 gene is a reasonable candidate for control of BMD, given its known physiological role and the effects upon BMD of mutations of this gene.

This chapter presents:

a) A review of PTHR1;

b) A high density linkage study of the PTHR1 locus;

c) Mutation screening of PTHR1 using denaturing high performance liquid chromatography (DHPLC);

d) Sequencing of mutations detected by DHPLC.

5.2 PTHR1 Review

5.2.1 PTHR1

PTHR1 is a G-protein coupled receptor, identified in 1991 by expression cloning of an opossum kidney cell cDNA library by Jüppner and colleagues (Jüppner et al. 1991). PTHR1 was recognised to be part of a unique and ancient G-protein linked receptor family due to its homology with the secretin and calcitonin receptors. This family (class II or family B G-protein coupled receptors) also includes receptors for vasoactive intestinal polypeptide types 1 and 2, gastric inhibitory polypeptide, corticotrophin releasing hormone, growth hormone releasing hormone, pituitary adenylate cyclase-activating peptide, glucagon-like peptide 1 and glucagon. The shared characteristics include seven membrane spanning domains, large extracellular N-terminal domains (including two conserved sites for N-linked glycosylation), 48 AA residues identical in all receptors (including 8 cysteine residues) with many other residues highly conserved, and cognate ligands of 26-46 AA residues in length (Jüppner 1994). PTHR1 is widely expressed, with highest levels found in kidney and bone (Jüppner et al. 1991).

G-proteins are the intracellular signal relaying mechanism for many receptors. G-proteins consist of an α subunit loosely associated with a dimer of a β and a γ subunit. In its inactive form, the α subunit is bound to both guanosine diphosphate (GDP) and the $\beta\gamma$ dimer. Ligand activation of the associated receptor results in release of GDP by the $\alpha\beta\gamma$ complex, allowing guanosine trisphosphate (GTP) to bind to the α subunit. The GTP-α subunit complex dissociates from the $\beta\gamma$ dimer, and either the complex or the dimer (or both) then activate/s downstream effectors. GTP is then hydrolysed back to GDP; the

GDP-α subunit complex then binds with the βγ dimer, preventing continuing stimulation of downstream activators (Farfel et al. 1999).

Jüppner identified cDNA for a 585 AA receptor that bound PTH and PTHrP with equal affinity (Jüppner et al. 1991). Schipani and colleagues demonstrated that human PTH/PTHrP receptor cDNA is identical in both kidney and osteoblast-like sarcoma cells, encoding a 593 AA receptor (Schipani et al. 1993). Further, there was only one major messenger RNA species identifiable by Northern blotting of total RNA from human bone and kidney tissues, suggesting a single PTH-PTHrP receptor present in the major tissues concerned with calcium metabolism (Schipani et al. 1993).

The PTHR1 gene is located on chromosome 3p. It spans 20 kb with 14 coding exons varying in size from 42 bp (M6, coding for one of the transmembrane domains) to more than 400 bp (T1, the intracellular C-terminal tail). A 10 kb intron separates exon S (coding for the signal peptide) from exons E1, E2, E3 and G coding for the N-terminal extracellular region. The portion of the gene encoding this region (157 AA) spans 15 kb. The 436 AA region of the transmembrane loops and the tail portion is encoded by a region spanning 4 kb, with exons separated by much smaller introns varying in size from 81-1000 bp (reviewed in (Schipani et al. 1995)).

In bone of normal individuals, PTHR1 mRNA is expressed in osteoclasts, osteoblasts, osteocytes, and bone marrow cells, although PTHR1 protein expression is not detectable in mature osteoclasts (Langub et al. 2001). PTHR1 is detectable on immature osteoclasts (N.Athanasou, personal communication) and on mature osteoclasts in diseased states such as secondary hyperparathyroidism (Langub et al. 2001). Although no significant

correlation was found between serum PTH levels and levels of PTHR1 protein expression on osteoclasts in secondary hyperparathyroidism, the percentage of osteoclasts expressing PTHR1 protein was related to the erosive depth of resorption pits, a function of osteoclast activity (Langub et al. 2001). Elderly patients frequently have mildly elevated levels of PTH due to secondary hyperparathyroidism (reviewed in (Bilezikian et al. 2000)). Thus increased expression of PTHR1 protein on osteoclasts, along with increased osteoclastic activity, may play a role in the development of osteoporosis. Of note also, administration of $1\alpha,25(OH)_2D_3$ results in downregulation of PTHR1 expression in osteoblasts and preosteoblasts (Amizuka et al. 2000).

5.2.2 Promoter regions of PTHR1

The 5' regulatory region of PTHR1 is complex. In humans, three promoter regions (P1, P2, P3) have been identified, resulting in differentially spliced transcripts in different tissues (Bettoun et al. 1998; Bettoun et al. 1997; Manen et al. 1998). In contrast, two promoter regions have been identified in mice: P1 activity is mainly localised to the kidney and accounts for 90% of renal PTHR1 transcripts, whereas P2 is more ubiquitously active (Amizuka et al. 1997; McCuaig et al. 1995). Homology between human and murine first and second promoter regions is high, with 73-4% homology for P1 and 92% homology for P2 (Bettoun et al. 1997). Despite this conservation, P1 activity in human kidneys is weak and most (80%) renal transcripts arise from P3 activity, resulting in a fusion exon of the promoter and the signal sequence exon. P3 is also broadly active in other human tissues (Bettoun et al. 1998). In humans and in mice

P2 is ubiquitously active. P2 activity has been detected even in the kidney but only in tubular epithelial cells (Bettoun et al. 1998).

P1 gives rise to transcripts containing two untranslated exons (U1 and U2), whereas P2 and P3 transcripts contain a single exon (U3 and U4 respectively).

P1 contains several possible transcription initiation sites over a 100 bp region, although these do not include a TATA box (a common transcription start sequence). Potentially, different initiation sites may result in transcripts with 5'UTR differing in their capacity to form stable stem-loop structures, which in turn may result in varying efficiency of translation (Bettoun et al. 1997; Yiu et al. 1994). In contrast to P2 and P3, P1 is not (G+C) rich (approximately 50%) (Minagawa et al. 2000).

P2 and P3 are highly (G+C) rich and lie in the same CpG island. Both P2 and P3 contain single initiation sites. Both proximal promoter regions contain sequence motifs recognized by a number of transcription factors including Sp1, ETF family and myc-associated zinc finger proteins (MAZ) but (in common with P1) do not contain a TATA box (Minagawa et al. 2000). Combinations of transcription factors (Sp1 and MAZ, or Sp1 and ETF) have been found necessary for activity of several (G+C) rich TATA less promoter regions (reviewed in (Minagawa et al. 2000)).

Despite these similarities, P2 and P3 regulation are distinct. As above, very little P2 activity is present in the kidney where P3-directed transcription dominates PTHR1 expression. Further, P2 is the only promoter region of PTHR1 active up to mid-gestation. P2-directed transcripts have been detected in kidneys, calvariae and long bones from

human foetuses aged from 12 and 19 weeks, an important period of organ development and skeletal ossification. During this time, neither P1 nor P3 activity were detectable. (Bettoun et al. 2000; Bettoun et al. 1998)

P2-directed transcripts may be differentially spliced between U3 and SS, resulting in 187 and 129 bp products. Both long and short transcripts are found in kidney, spleen and skin fibroblasts, whilst only the short UTR was found in human osteoblast-like osteosarcoma cell lines. The shorter UTR has a less stable stem loop with less secondary structure formation. As extensive secondary structure can inhibit initiation of translation, it may be that the shorter U3 is more efficiently translated (Bettoun et al. 1998).

5.2.3 Interaction of PTHR1 with PTH and PTHrP

The structure and function of PTHR1 have recently been reviewed (Goltzman 1999; Mannstadt et al. 1999). PTHR1 is activated by both PTH and PTHrP. Both ligands bind with equal affinity to PTHR1 and elicit a robust and sensitive cAMP second messenger response through the adenyl cyclase A/protein kinase A pathway. An additional second messenger response of inositol trisphosphate (IP_3) is elicited through activation of the phospholipase C/protein kinase C pathway (Abou-Samra et al. 1992), though this is approximately 10-fold less sensitive than the adenyl cyclase pathway (Takasu et al. 1999). Cell surface density of receptor is a key determinant of the magnitudes of cAMP and IP_3 responses to PTH and PTHrP. Thus regulation of PTHR1 expression modulates the character and intensity of response to ligand binding (Takasu et al. 1999).

Synthetic N-terminal peptide hormone analogues PTH^{1-34} and $PTHrP^{1-34}$ are both critical and sufficient for binding of PTHR1 and activation of the cAMP signalling system. The midregion and C-terminal portions of intact PTH and PTHrP are not thought to interact with PTHR1. Homology between PTH^{1-34} and $PTHrP^{1-34}$ is greatest at the N-terminal region, with eight identical amino acids in the region 1-13. Subsequently homology between the two diminishes, with only three identical amino acids in the region 14-34.

Structural studies of PTH and PTHrP had suggested that PTH^{1-34} and $PTHrP^{1-34}$ contained both an N-terminal and a C-terminal helix, with a flexible hinged midregion. Receptor binding and activation were thought to be a function of helical stability. However, recent three-dimensional X-ray crystallography of PTH^{1-34} revealed its structure as a slightly bent long single α-helix, although residues 6-20 and 21-33 form two distinct amphiphilic helices (Jin et al. 2000). As yet there has been no definitive X-ray crystallography of PTHrP, PTHR1, or the ligand-receptor interaction with PTHR1.

The C-terminal regions of the analogues (i.e. PTH^{14-34} and $PTHrP^{14-34}$) contain the main receptor-binding domain and interact with the N-terminal extracellular domain of PTHR1 (Jüppner et al. 1994). Although the C-terminal regions of the analogues PTH^{1-34} and $PTHrP^{1-34}$ lack amino acid homology, they both form similar amphiphilic helices that bind with similar high avidity to PTHR1 (Neugebauer et al. 1992; Pellegrini et al. 1998). Binding of ligand to receptor also involves interactions with the membrane-spanning helices and extracellular loops of the receptor.

The N-terminal parts of PTH and PTHrP interact with the membrane-spanning helices and loops of the receptor and are critical for activation of the adenyl cyclase system.

Extension or deletion of the N-terminal part of the PTH^{1-34} and $PTHrP^{1-34}$ markedly inhibits the capacity of either to increase cAMP (Goltzman 1999).

PTHR1 activation of the phospholipase C/IP_3 second messenger system is poorly understood. The C-terminal part of PTH^{1-34} is thought to contain the phospholipase C stimulating domain. N-terminally truncated PTH forms fail to stimulate adenylate cyclase and lack anabolic activity, but retain the capacity to stimulate phospholipase C. Thus the cAMP second messenger system may be essential (though not necessarily sufficient) for anabolic activity.

The intracellular loops and C-terminal portions of PTHR1 are linked with G-protein α subunits, in particular the second intracellular loop. Stimulation of cAMP and IP_3 second messenger systems may rely upon activation of different $G\alpha$ subunits.

In addition to its N-terminal interaction with PTHR1, PTHrP residues 87-107 may also act as a nucleolar targeting signal (Amizuka et al. 2000).

5.2.4 The Role of PTHR1 in Embryogenesis: Interaction with PTHrP

Bone development occurs in two distinct developmental patterns - endochondral ossification, in which a cartilage mould is replaced by bone (the mechanism for most bone formation), and intramembranous ossification, in which bone matrix is deposited directly by osteoblasts without such a mould. During endochondral ossification, mesenchymal cells differentiate to become chondroblasts: these then secrete cartilaginous matrix and differentiate into chondrocytes. The chondrocytes of the future midshaft proliferate, hypertrophy and synthesize a distinct extracellular matrix. This hypertrophic

matrix allows osteoclast invasion. Osteoclasts arise from haematopoietic stem cells and resorb the hypertrophic matrix, allowing angiogenesis and formation of the bone marrow. Osteoblasts (derived from mesenchymal stem cells) bind to the cartilaginous matrix remnants and deposit bone matrix into their surrounds. The residual cartilage remnants are later resorbed and replaced by bone. At the ends of the bone shaft, the epiphyseal growth plates are formed, where chondrocytes proliferate, hypertrophy and eventually undergo apoptosis. Additionally, intramembranous ossification also takes place in the midshaft area, in the perichondrium to form a 'bone collar'. The first bone formed is woven bone and the resulting trabeculae are known as the primary spongiosa; subsequent remodelling results in replacement of the woven bone and the cartilaginous remnants with lamellar bone, resulting in the mature state of trabecular bone called secondary spongiosa (reviewed in (Lanske et al. 1999)).

Locally acting negative feedback loops involving PTHR1, PTHrP and Indian hedgehog (Ihh) regulate the rate of chondrocyte differentiation in the growth plate. Chondrocytes express Ihh as they make the transition from the proliferative to hypertrophic stages. Ihh increases expression of PTHrP in the periarticular perichondrium. PTHrP binds to PTHR1 expressed on proliferating chondrocytes, inhibiting hypertrophic differentiation, maintaining chondrocyte proliferation, and preventing production of more Ihh-producing cells (Karp et al. 2000). Thus the level for PTHrP expression critically determines the rate of chondrocyte differentiation. There are also spatial differences in PTHrP expression resulting in polarity of PTHrP expression (greater levels of PTHrP at the ends of the bones). At least in part this is responsible for the normal polarity of chondrocyte differentiation and long bone development (Lanske et al. 1999).

5.2.5 Knockout Mouse Models for PTHR1, PTH and PTHrP

PTHR1, PTH and PTHrP homozygous (-/-) knockout mice share a phenotype of short-limbed dwarfism and abnormal endochondral bone formation (Karaplis et al. 1994; Karp et al. 2000). The mice exhibit acceleration of chondrocyte differentiation (decreased proliferation and extensive hypertrophy) and accelerated ossification. However there are additional abnormalities specific for each mouse strain. The differences between PTHR1 (-/-) knockout mice and PTHrP (-/-) knockout mice may be due to the ability of PTH to partially overcome the deficiency of PTHrP. Of note, PTHR1 (-/-) knockout mice usually die midgestation and in addition to endochondral abnormalities have marked reduction of trabecular bone formation (Lanske et al. 1999). PTHrP homozygous (-/-) knockout mice usually die postnatally, thought to be due to asphyxia (Karaplis et al. 1994), however heterozygous (+/-) PTHrP knockout mice reach adulthood and develop osteopaenia (Amizuka et al. 2000).

5.2.6 PTHR1 Mutations in Humans

Jansen's metaphyseal chondrodysplasia is an autosomal dominant form of short-limbed dwarfism with abnormal growth plate maturation. It is due to constitutively activated PTHR1 with adenylate cyclase activation in the absence of ligand binding. Disease features resemble primary hyperparathyroidism, with increased bone turnover, multiple fractures, low bone mineral density, hypercalcaemia and hypophosphataemia but with normal to undetectable levels of PTH and PTHrP (Parfitt et al. 1996).

Several mutations of PTHR1 resulting in Jansen's metaphyseal chondrodysplasia have been described. These include mutations of:

- the second transmembrane domain (arginine substituted for histadine at residue 223) (Schipani et al. 1995)
- the sixth transmembrane domain (proline substituted for tryptophan at residue 410) (Schipani et al. 1996)
- the seventh transmembrane domain (isoleucine to arginine at residue 458) (Schipani et al. 1999).

Expression of the mutant receptor by transfected COS cells (at approximately 30% of normal maximal cell-surface expression) resulted in 5-8 fold increases in intracellular cAMP although IP_3 levels were unchanged. (Schipani et al. 1999).

Of note, not all patients with Jansen's disease have been found to have mutations of PTHR1. Such patients have been thought to have somatic mosaicism affecting primarily the growth plate cartilage (Schipani et al. 1996).

Transgenic mice carrying the mutant human PTHR1 (His223 to Arg), with expression targeted to the growth plate, have a significant deceleration of chondrocyte differentiation and delayed bony mineralization (Schipani et al. 1997). This phenotype has also been noted in transgenic mice overexpressing the PTHrP gene (Weir et al. 1996). Additionally, targeted expression of constitutively overactive PTHR1 overcame the skeletal abnormalities observed in PTHrP (-/-) knockout mice (Schipani et al. 1997).

Blomstrand's chondrodysplasia is a lethal autosomal recessive disorder resulting in advanced bone maturation, accelerated chondrocyte differentiation, premature ossification and probable abnormal mineral ion homeostasis (Blomstrand et al. 1985). Several cases have been described in the literature. The first analysed case resulted from deletion of 11 amino acids from the fifth transmembrane domain, due to a point mutation (G to A) causing a novel splice site. This resulted in abnormally spliced maternal mRNA; the paternal allele was silent for unknown reasons (Jobert et al. 1998). A second case resulted from a consanguineous marriage in which the offspring had a homozygous missense mutation in the extracellular N-terminal domain in exon E3 resulting in a leucine substituted for proline at residue 132 (Karaplis et al. 1998). Although the mutant PTHR1 was expressed in both these cases, there was abnormal PTH and PTHrP binding with reduced cAMP activation and (at least in the second case) undetectable PTH-induced IP_3 changes. A further case, again resulting from a consanguineous marriage, was due to a homozygous point mutation at position 1122 with loss of a guanine residue. This affected the second extracellular loop (EL-2) resulting in a shift in the reading frame and a truncated protein that completely diverged from the wild-type sequence after AA 364; thus the mutant PTHR1 lacked transmembrane regions 5,6,7, the interconnecting loops and the C-terminal tail. Although mutant mRNA was expressed, there was no response to PTH challenge with no cAMP increment (Karperien et al. 1999).

5.2.7 PTHR2

The PTH receptor type 2 (PTHR2) is 51% homologous with PTHR1 and responds to PTH with activation of the cAMP second messenger system. It is unresponsive to PTHrP

(Usdin et al. 1995). Unlike the ubiquitous expression of PTHR1, PTHR2 tissue expression is limited. PTHR2 mRNA is found predominantly in the brain, but also in the exocrine pancreas, testis, placenta, arterial and cardiac endothelium, vascular smooth muscles and the lungs (both bronchi and parenchyma) (Usdin et al. 1996). Its biological action is currently unknown. The locus for PTHR2 is also unknown.

5.3 Fine Mapping of the PTHR1-Containing Region

5.3.1 Introduction

Having demonstrated linkage of BMD with two markers, further mapping is needed to determine the gene(s) responsible for the observed linkage. Before investigating the PTHR1 locus as a suitable candidate gene between the two markers, it was important to ensure that the demonstrated linkage at d3s1289 and d3s3559 was not due to chance characteristics of these markers alone, and to establish boundaries for the area of linkage. Therefore a further set of markers from chromosome 3p was amplified, genotyped and analysed for linkage with BMD using the same families that were used in Chapter 4.

5.3.2 Methods

Fourteen additional microsatellite markers in the area of observed linkage were selected, using the Whitehead Institute STS database (http://www-genome.wi.mit.edu) to establish chromosomal position. The markers selected are presented in table 5.3.2, with genetic distance in Kosambi cM. Markers were amplified, separated and genotyped as described in Chapter 2, using the same families as the studies in Chapter 4.

Table 5.3.2: Markers used for further linkage mapping of PTHR1 region

Marker name	Position in cM from p-telomere
D3S1266	46.9
D3S1768	56.1
D3S1298	56.7
D3S3559	62.7
D3S3647	65.1
D3S2420	67.9
D3S3640	67.9
D3S2384	67.9
D3S1578	67.9
D3S1289	69.1
D3S1582	69.6
D3S1606	72.9
D3S3621	74.1
D3S1547	77.4
D3S1600	85.7
D3S3571	90.1

5.3.3 Statistical Analysis

Allele numbers and frequencies were calculated from the observed data. BMD was expressed as z-scores (adjusted for age and gender). BMD was also adjusted for effects of body size by regressing BMD against BMI.

Given the results of the comparison between SOLAR and MapMaker/Sibs (presented in Chapter 4), the data were analysed in nuclear families using MapMaker/Sibs (Kruglyak et al. 1995). As previously, the original 115 families were divided into 165 nuclear units containing at least one sibling pair for analysis, using the programme 'GAS' (A. Young, unpublished). Nonparametric two-point and multipoint linkage analyses were performed using both Haseman-Elston (traditional and expectation maximisation methods) and variance components analysis. Analyses were performed both with and without correction for the number of sibling pairs drawn from each family (as described in Chapter 4). Single results presented below are for the 'all pairs' setting both with and without correction. Where a range of LOD scores is presented, the lower value is for the 'all pairs' setting with correction, and the higher value for no correction.

5.3.4 Results

Two point linkage results of additional microsatellite markers on chromosome 3 and BMD at both LS and FN are presented in Table 5.3.4. Multipoint linkage results are shown graphically in Diagram 5.3.4. All results refer to variance components analysis as in all cases this was more significant than the corresponding Haseman-Elston analyses (both traditional and expectation maximisation methods).

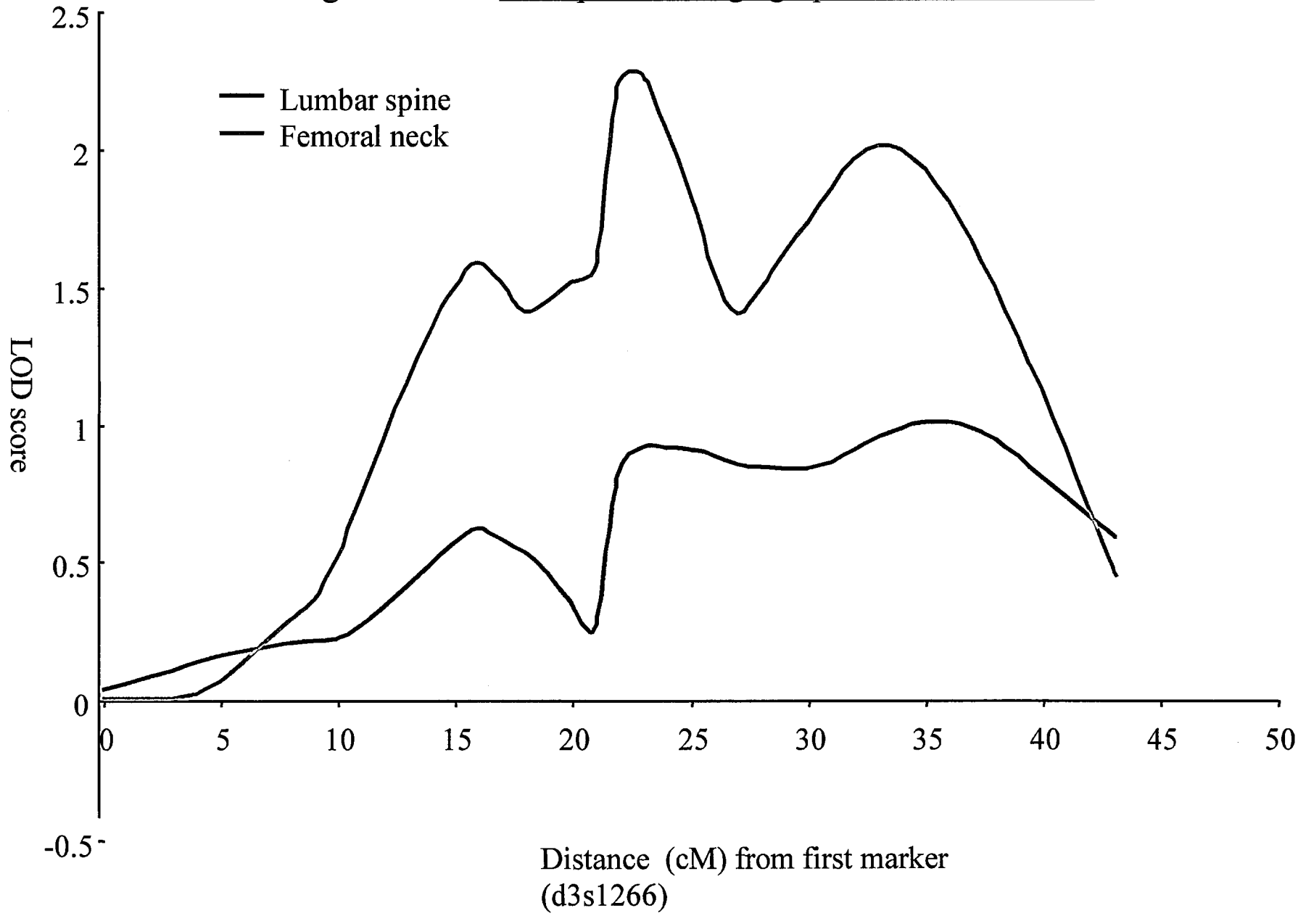
Table 5.3.4: Two Point LOD Scores for Additional Markers in PTHR1 region

Microsatellite marker position is measured from the first marker used.

Correction is for number of sibling pairs drawn from each family, and is over-conservative.

Marker number	Marker name	Relative position (Kosambi cM)	Results at LS		Results at FN	
			Without correction	With correction	Without correction	With correction
1	D3S1266	0	0.000	0.000	0.000	0.000
2	D3S1768	9.2	0.000	0.000	0.05615	0.1648
3	D3S1298	9.8	0.05494	0.1269	0.07420	0.1006
4	D3S3559	15.8	1.467	1.224	2.179	1.498
5	D3S3647	18.2	0.000	0.01190	0.09708	0.1086
6	D3S2420	21	0.000	0.002797	0.1853	0.1640
7	D3S3640	21	0.000	0.000	0.000	0.000
8	D3S2384	21	0.5290	0.5343	0.6294	0.6088
9	D3S1578	21	0.2348	0.2452	0.2645	0.2955
10	D3S1289	22.2	0.4107	0.2869	3.501	2.698
11	D3S1582	22.7	0.6578	0.5580	1.849	1.669
12	D3S1606	26	1.494	1.1848	0.1459	0.2435
13	D3S3621	27.2	0.4552	0.3285	0.1010	0.02523
14	D3S1547	30.5	1.246	0.8179	2.447	1.554
15	D3S1600	38.8	0.9081	0.5989	1.108	0.7084
16	D3S3571	43.2	0.2825	0.2526	0.2770	0.2500

Diagram 5.3.4: Multipoint linkage graph for PTHR1 locus



5.3.5 Discussion

Additional linkage mapping with further markers on chromosome 3 established that the PTHR1 locus was within the boundaries of the observed area of linkage with BMD. However this does not establish the gene/s responsible for the observed linkage.

Linkage mapping is inefficient in identifying genes determining complex traits and may be only able to isolate a disease-causing gene to an area as broad as 1 cM (depending upon the relative risk of disease conferred by that gene and the number of sibling pairs sampled) (Kruglyak et al. 1995). To localise a gene causing a five-fold increase in risk of a qualitative disease to an offspring even to a 1 cM chromosomal area requires on average 200 sibling pairs, whilst for a locus increasing risk two-fold 700 sibling pairs are needed (Kruglyak et al. 1995). The figures for quantitative traits are not known. Roberts and colleagues have suggested that for sample sizes of the order used in this study, the confidence intervals for locating a gene relative to the point of maximal multipoint linkage extend tens of cM either side of the maximum LOD score (Roberts et al. 1999). Further, Terwilliger and colleagues have shown with simulation studies that true peaks tend to be longer than false peaks (Terwilliger et al. 1997). It is thus improbable that linkage mapping will 'pinpoint' a disease-causing gene. Therefore to further isolate such loci, other approaches are needed, which may include association studies in both families and the general population.

Linkage varied markedly between the markers examined, even for adjacent markers, and the multipoint curve did not contain a single sharp peak. This is not usual in linkage studies, as mentioned above. Observed linkage depends on many factors including the

polymorphism information content of the markers chosen (discussed in Chapter 2), chance variation in allele sharing of the markers chosen within the families in the sample, and ancestral haplotypic effects.

So in summary, further linkage mapping confirmed the boundaries for the area of linkage observed on chromosome 3p. The area included the PTHR1 locus. It was therefore reasonable to study this gene further in association studies, to assess if polymorphisms of PTHR1 were responsible for the population variance of BMD.

5.4 Mutation Screening of the PTHR1 Locus

5.4.1 Introduction

The candidate gene study presented in Chapter 4 and the above fine mapping study demonstrated linkage of BMD to the chromosomal area containing the PTHR1 locus. Polymorphisms of this gene and the promoter regions were therefore sought using DHPLC as an efficient means of screening for mutations. Individual samples containing polymorphisms could then be sequenced to establish exact base changes and RFLP assays designed for efficient genotyping in larger scale family- and population-based association studies.

5.4.2 Methods

5.4.2.1 Family Members

Samples from 36 unrelated patients with low BMD at FN were selected from the family cohort. Mean femoral neck z-score was –2.45 (range –3.31 to –1.96). 23 samples were from women, 13 from men.

5.4.2.2 Mutation Screening of the PTHR1 Locus using DHPLC

Heteroduplex detection with DHPLC was used to screen for the presence of polymorphisms in the 14 coding exons, intron-exon boundaries and the promoter regions of PTHR1. This method is discussed in detail in Chapter 2. Primer sequences for this work were obtained from previous publications (Schipani et al. 1995) (Bettoun et al. 1997) or were redesigned using the available published sequence (http://www.ncbi.nlm.nih.gov/Genbank). Primers were synthesized by Gibco/Life Technologies™, Paisley, UK. Primer sequences and PCR conditions are listed in Appendix 5.4.2.

5.4.2.3 Sequencing

Exons containing heteroduplexes were sequenced to identify the polymorphisms responsible. Sequencing was performed as described in Chapter 2. Sequencing data was compared with previously published sequence (Bettoun et al. 1998; Bettoun et al. 1997; Schipani et al. 1995) and with genomic sequence released by the Human Genome Project

(http://genome.uscs.edu), using the programmes BLAST 2 (Tatusova et al. 1999) and Sequence Navigator™ (Perkin Elmer, Applied Biosystems, Warrington, UK).

5.4.3 Results

Heteroduplexes were detected in three exons. Heteroduplexes of the M6-7 fragment were detected in 22/36 samples (Diagram 5.4.3.1) and of the T1 fragment in 2/36 samples (Diagram 5.4.3.2). Heterozygosity was also demonstrated in the U3 fragment (second promoter region), detected in 4/36 samples (Diagram 5.4.3.3).

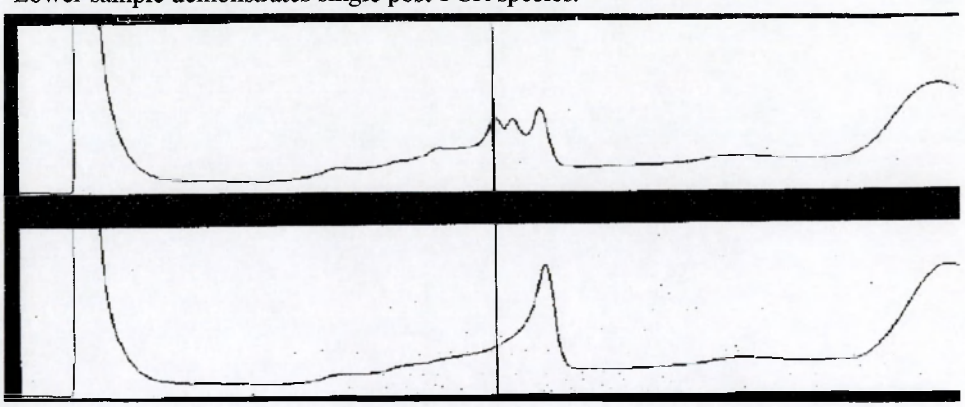
U4 amplification to the standard required for DHPLC was unsuccessful, despite multiple primer redesign (including nested primers), magnesium and annealing temperature titration experiments, de-aza dGTP nucleotide substitution, addition of DMSO and use of Q solution (Qiagen, Crawley, West Sussex).

Sequencing of the M6-7, T1 and U3 fragments demonstrated the polymorphic bases responsible for heteroduplexes. These are shown in Diagrams 5.4.3.1, 5.4.3.2 and 5.4.3.3 respectively. Numbering of bases is from sequence obtained using the primers listed in Appendix 5.2. Numbering of amino acids is from SWISS-PROT database (http://www.expasy.ch/cgi-bin/niceprot.pl?Q03431).

Diagram 5.4.3.1: Mutation detection of M6-7 exon

1. Mutation detection with DHPLC

Upper sample demonstrates presence of heteroduplexes
Lower sample demonstrates single post-PCR species.



2. Sequencing of M6-7 exon: C/T polymorphism

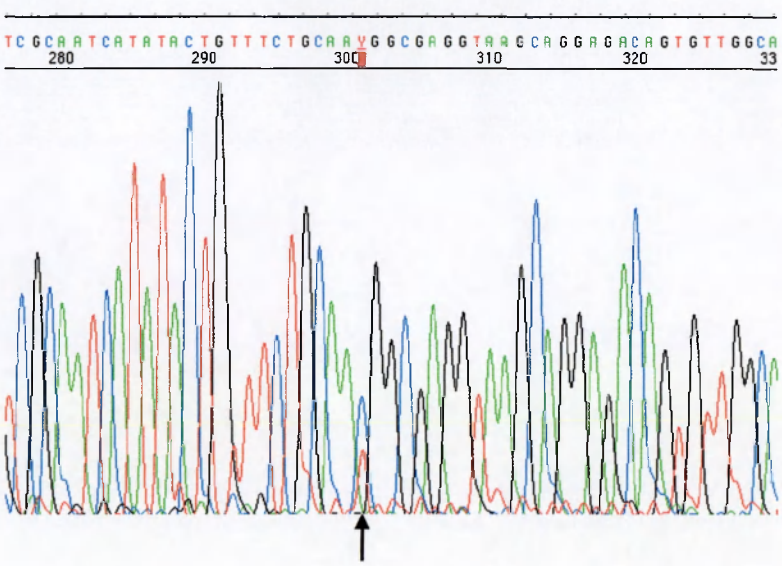
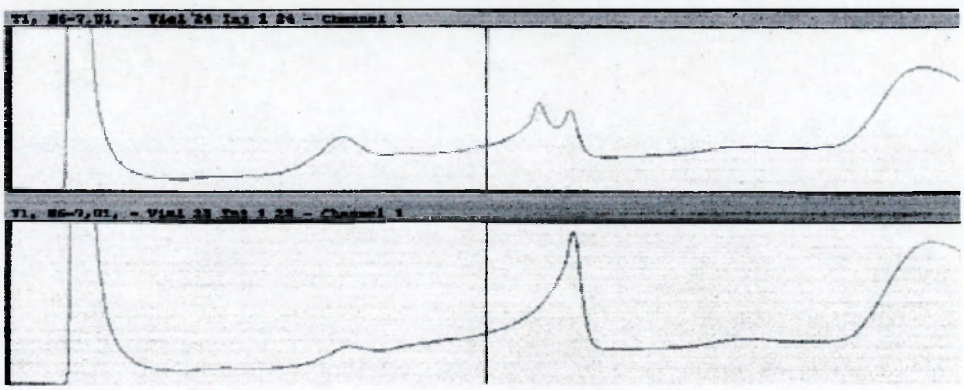


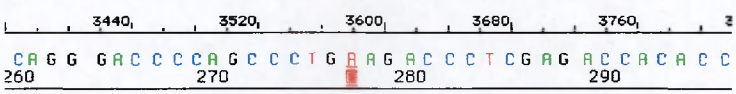
Diagram 5.4.3.2: Mutation screening of exons T1-2

1. Mutation detection with DHPLC

Upper sample demonstrates presence of heteroduplexes
Lower sample demonstrates single post-PCR species.



2. Sequencing of T1-2 exon: A/G polymorphism



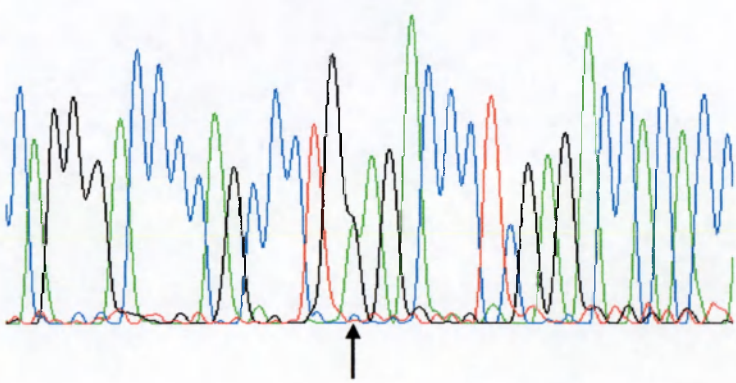
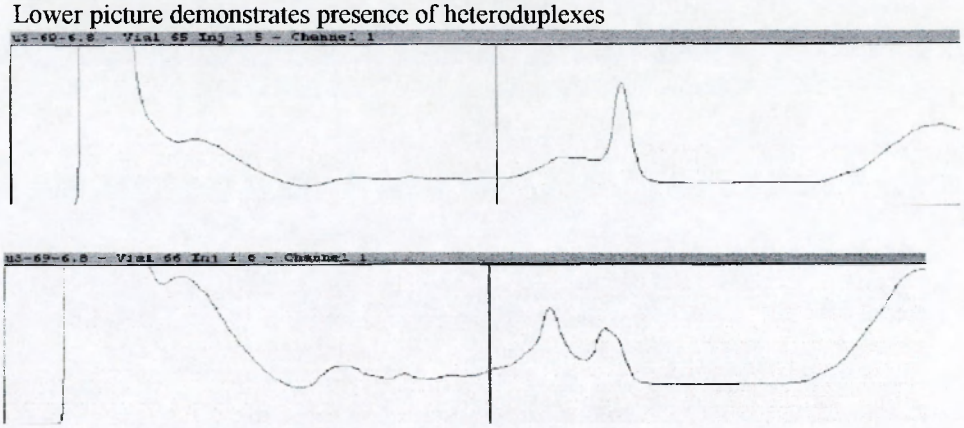


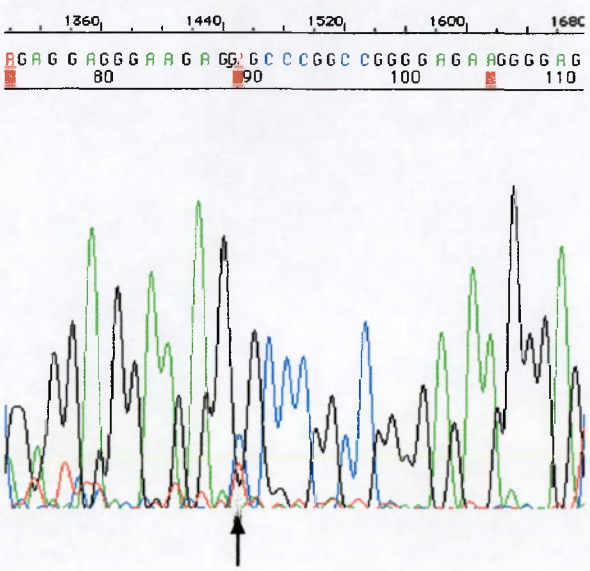
Diagram 5.4.3.3: Mutation screening of exon U3

1. Mutation detection with DHPLC

Upper picture demonstrates single post-PCR species
Lower picture demonstrates presence of heteroduplexes



2. Sequencing of U3 exon: C/T polymorphism



M6-7 Fragment

A C/T bp change at position 301 (in exon M7) was responsible for the observed heteroduplexes.

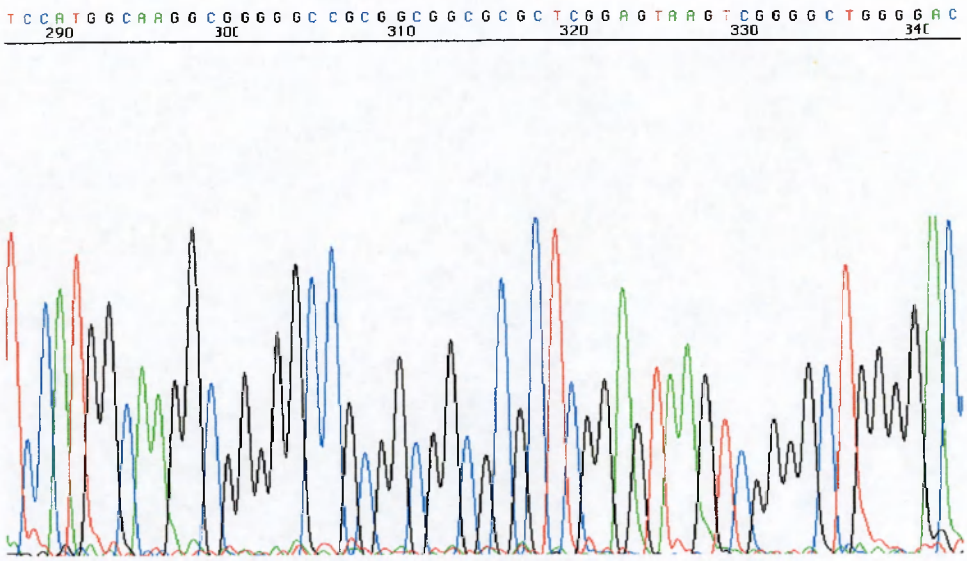
T1 Fragment

An A/G bp change at position 277 was found, resulting in a coding change in AA 546 (Glu to Lys).

U3 Fragment

A novel polymorphism was found at position 89, with a C/T bp change. Further, sequence of this promoter region from both heterozygote and homozygote samples showed several consistent differences with the previously published sequence (Bettoun et al. 1997). When compared with genomic sequence data published by the Human Genome Project (http://genome.ucsc.edu), the sequencing obtained in these experiments was a closer match than the previously published sequence (Diagram 5.4.3.4). Sequences were compared using the 'BLAST2' programme (http://www.ncbi.nih.gov/blast.bl2seq/wblast2.cgi) with results shown in Appendix 5.4.3.

Diagram 5.4.3.4:
Sequencing of U3 exon – area of discrepancy compared with previously published sequence



5.4.4 Discussion

DHPLC screening of the 14 coding exons and three of the promoter region exons for PTHR1 in 36 subjects with low femoral neck BMD showed evidence of polymorphisms in three exons. Polymorphisms had previously been demonstrated in both M7 and T1 exons (Hustmyer et al. 1993) (Schipani et al. 1995). The polymorphism demonstrated in U3 is a novel finding.

Although ablation of PTHR1 clearly results in a severe and lethal phenotype in mice and humans, more subtle polymorphic differences in the coding regions could result in population variability in PTH and PTHrP binding and their ability to elicit cAMP and IP_3 responses. Additionally, alterations of the promoter regions could result in abnormal splicing, stability or translation of PTHR1 mRNA, and, given the tissue specificity of the different promoters, such alterations in PTHR1 expression might be site-specific.

Apparently benign polymorphisms of PTHR1 have been previously reported in the literature. In searching for the molecular basis of pseudohypoparathyroidism type 1b, Schipani and colleagues found silent polymorphisms in exons G (1/17), M4 (1/17), and M7 (15/17) (Schipani et al. 1995). Base changes were found affecting two introns: one at the splice-donor site of the intron between E2 and E3, and one between G and M1. The mutation of the E2/E3 splice-donor site did not affect total mRNA production from COS7 cells. A missense mutation was found in the cytoplasmic tail of PTHR1 in exon T1, at residue 546 in 3/17 patients and 1/60 healthy individuals, resulting in substitution of Glu for Lys at AA 546. However, ligand binding affinity, PTH^{1-34} induced cAMP and phosphoinostitol turnover in cells transfected with the mutant receptor were

indistinguishable from cells transfected with wild-type receptor (Schipani et al. 1995). (Of note, the molecular basis of pseudohypoparathyroidism type 1b has subsequently been shown to arise from abnormalities of the paternally imprinted, renally expressed $G_S\alpha$ subunit encoded at chromosome 20q13 (Jüppner et al. 1998)).

The M7 exon polymorphism detected in the osteoporotic population used in this study has also been observed in Caucasian, Black and Asian populations (Hustmyer et al. 1993). The frequency of heterozygotes in the osteoporotic group (22/36) appeared excessive. When assessed by Fisher's exact test there was no significant deviation from Hardy-Weinberg equilibrium ($p=0.17$). Although this polymorphism does not result in an amino acid coding change, it may be in linkage disequilibrium with a polymorphism or mutation with functional effect. This mechanism is thought to underlie the association of intronic VDR polymorphisms and BMD (reviewed in Chapter 1).

The intracellular C-terminal tail of PTHR1, along with intracellular loops of the transmembrane domains, is thought to interact with G protein α subunits. *In vitro* functional studies of the missense mutation of exon T1 did not demonstrate any difference from wild-type receptor. However, *in vivo* studies of the effects of carriage of this mutation upon PTHR1 G-protein signalling have not been performed.

As discussed above, the second promoter region of PTHR1 is ubiquitously active in humans. In mice it is the only promoter region determining PTHR1 transcription in bone and cartilage cells, where it is differentially regulated by $1,25(OH)_2D_3$ (Amizuka et al. 1999). The polymorphism found in U3, coding for the second promoter region, may result in alteration of PTHR1 transcription in bone and cartilage cells.

Minagawa and colleagues recently analysed the P2 and P3 promoter sequences by progressive deletion to determine the critical minimum areas for promoter activity. In both P2 and P3, proximal sequences within 100bp of the transcriptional start site were found to contain the critical region for activity. Truncation of P2 between –91 and –12bp of the transcriptional start site abolished P2 activity. Regulation of P3 activity was more complicated. Progressive deletion of P3 from –842 to –147 led to a 2-fold loss of P3 activity. Truncation of P3 from –115 to +42 also diminished P3 activity. However, deletion of an adenosine-rich region (ARR) from –147 to –115 bp increased P3 activity, suggesting that this region contains a repressor of activity. When the ARR was inserted upstream of P2, it resulted in diminished P2 activity also. In contrast, increased activity was observed when the ARR was inserted into a truncated thymidine kinase promoter. Apart from the ARR, P2 and P3 are very similar in sequence and organisation (Minagawa et al. 2000).

The polymorphism demonstrated in U3 (i.e. P2) lies within the critical region for P2 activation (–32bp from the transcriptional start site). Multiple transcriptional factors bind to this region with synergistic effects upon promoter region activation. The detected polymorphism may alter transcriptional activation of the second promoter region, through effects upon transcriptional factor binding.

PTHR1 expression *in utero* is under the control of the second promoter region, at least until midgestation (Bettoun et al. 1998). Alteration of P2 function during this critical period of skeletal development could have long ranging effects upon bone density.

Mutation screening of the third promoter region by DHPLC was unsuccessful. Therefore, possible polymorphisms in this region would have been missed. Subsequent release of genomic sequence in September 2000 by the Human Genome Project (http://genome.ucsc.edu) revealed several discrepancies with previously published sequence (Bettoun et al. 1998) (see Appendix 5.4.4) illustrating how difficult this area is to sequence. The genomic sequence identified by the Human Genome Project may have helped design more effective primers for amplification of this highly (G+C) rich area to the standard required for DHPLC. This will be the subject of future work.

The PTHR1 locus is a large gene, spanning 20 kB. This study only examined the 14 coding exons, the intron/exon boundaries and two of the three 5'UTR promoter regions. Further intronic polymorphisms will have been missed. Indeed, several have been identified by SNP consortiums (http://snp.cshl.org/snp and http://www.ncbi.nlm.nih.gov/SNP).

In summary, several polymorphisms of PTHR1 were detected in a small population of osteoporotic patients, including a novel polymorphism of the second promoter region. Further investigation of these polymorphisms in a larger population will determine if an association exists between any of these polymorphisms, population variance in PTHR1 activity and BMD.

Chapter 6: Association Studies of the COL1A1 Sp1 Binding Site Polymorphism

6.1 Introduction

There is mounting evidence that alleles of COL1A1 contribute to the population variance of BMD. Several cross-sectional (Grant et al. 1996; Keen et al. 1999; Langdahl et al. 1998; Uitterlinden et al. 1998) and some longitudinal (Harris et al. 2000) studies of a polymorphism in an Sp1 binding site in the first intron of the COL1A1 gene have demonstrated an association with low BMD in both men and women, in several Caucasian populations. Negative association studies have also been published (Garnero et al. 1998; Heegaard et al. 2000; Hustmyer et al. 1999; Liden et al. 1998; Sowers et al. 1999). Some evidence of linkage of this area with BMD was demonstrated in the candidate gene study presented in Chapter 4, with maximal LOD scores of 1.7 at femoral neck and 0.5 at lumbar spine. Moderate linkage of the COL1A1 locus at the femoral neck, but not the lumbar spine, was also observed in an Australian twin and family study (Brown et al. 2001).

In this chapter, two different association studies of the COL1A1 polymorphism in a British Caucasian population are presented. Firstly, a population study for association of the COL1A1 polymorphism was performed on unrelated individuals drawn from the family collection. Secondly, association was assessed by a within-family association method, using quantitative transmission disequilibrium statistics (QTDT).

TDT is less powerful in detecting association than are population case-control association studies (Morton et al. 1998). However the latter are sensitive to population stratification

(see introduction). Population studies cannot assess for the presence of imprinting, and, given the genetic epidemiology results presented in Chapter 3, an *a priori* assumption of imprinting – affecting at least some of the genes determining BMD – is reasonable.

6.2 A Population Association Study of Alleles Of COL1A1 with BMD

The aim of this study was to assess the role of polymorphisms at an Sp1 binding site of the COL1A1 gene in determining BMD in a cohort of unrelated individuals.

6.2.1 Participants

155 unrelated individuals were identified from the family collection (see Chapter 3). Either probands or family founders were selected. BMD was measured by DXA at both lumbar spine and femoral neck.

6.2.2 Genotyping at the COL1A1 Locus

RFLP typing for the Sp1 binding site polymorphism of COL1A1 was performed using previously published methods (Grant et al. 1996) but with an isoschizomer for the *Ball* restriction enzyme. PCR was performed with mismatched primers to introduce an RFLP site for the restriction enzyme on the polymorphism-carrying allele. The forward primer was 5'- CCCGACACCTAGTGGCCGT-3' and the reverse primer was 5'-GGAGAGAAGGGAGGTCCATCCCTCATCCTGGCC-3', containing mismatched bases to introduce the RFLP site. PCR was performed using 20ng of DNA template, with 1 unit AmpliTaq Gold (Perkin Elmer, Boston, Massachusetts, USA), 1xPCR Buffer

(Perkin Elmer, Boston, Massachusetts), 25μM dNTPs, 1.5μmol/L $MgCl_2$, 0.5μmol/L each of forward and reverse primer in a final volume of 20μL. PCR conditions were 12 minutes at 95° C to activate the AmpliTaq Gold (Applied Biosystems, Warrington, UK), followed by 35 cycles of [(30 seconds at 94°C), (30 seconds at 56°C), (30 seconds at 72°C)], ending with 10 minutes at 72°C.

The PCR product size was 321 bp. The product was digested with *MscI* enzyme (New England Biolabs (UK) Ltd, Hitchin, Hertfordshire, UK) for 1 hour at 37°C. Samples were run out on a 2% Agarose gel. The rare polymorphism ('s' allele) is cleaved to give 290 and 31 bp fragments, the uncut allele ('S') remains 321 bp.

6.2.3 Statistical Methods

BMD of three genotypic groups was compared by MANOVA using the statistical package SUPERANOVA Version 1.11 (Abacus Concepts, Berkeley, California). Analysis of variance was used to compare the variances of the three genotype groups. Variance of BMD for each genotype group was compared with variance between each genotype group. The total variance of the three groups (the sum of the squared difference of each measurement from the mean, hence known as sum of squares) is split into the sum of squares due to differences between the three genotypes, and the sum of squares due to differences within each group (residual sum of squares). The amount of variation per degree of freedom (d.f.) is calculated as the mean square. The mean squares are compared by the F test or variance ratio test. In the case of the null hypothesis (i.e. no association between BMD and genotype) then the variance within the groups would be approximately the same as the variance between groups and the F test would be

approximately one (Kirkwood 1988). Where association does exist, F will be greater than one.

$$F = \text{between groups MS/within groups MS, d.f.} = \text{d.f.}_{\text{between groups}}, \text{d.f.}_{\text{within groups}}$$

$$= (k-1), (N-k)$$

where k = number of groups

and N = total number of observations.

When the groups to be compared differ only by one factor then one-way analysis of variance can be used. If the groups may be subdivided by several different factors or if it is important to account for strong sources of variation, then either multiple analyses of variance or multiple regressions can be used, and are equivalent methods (Kirkwood 1988).

Age, gender, height and weight were used as covariates in this multiple regression analysis. Additionally, the cohort was stratified into tertiles according to age and the analysis repeated. This analysis may be more powerful when the relationship between covariates is not strictly linear.

6.2.4 Results

155 people (73 males, 82 females) of mean age 60.7 years were used in this association analysis.

The mean BMD z-score of the population was –0.83 at lumbar spine and –0.81 at femoral neck.

Genotype frequencies were calculated by direct counting. Expected genotype frequencies were calculated using the formula:

$$p^2+2pq+q^2=1$$

where p^2 and q^2 refer to the respective homozygous genotype frequencies and 2pq is the heterozygous genotype frequency. The expected and observed genotype frequencies were compared using the chi-squared statistic.

Mendel's first law of genetics is that alleles at a locus segregate independently from one another. As a consequence, frequencies of genotypes and of alleles remain constant from one generation to the next, a property known as Hardy-Weinberg equilibrium. The conditions necessary to maintain the Hardy-Weinberg equilibrium are: random mating, no migration into or out of the population, no inbreeding, no selective survivorship among genotypes, large population sizes (to avoid sampling errors), and no mutation or other force that would alter allele frequencies (Khoury et al. 1993). The genotype frequencies of this population were 0.16 for the 's' allele and 0.84 for the 'S' allele and were in Hardy-Weinberg equilibrium ($p = 0.17$ to 1). The allele frequencies in this cohort were not dissimilar to those previously published (Grant et al. 1996; Keen et al. 1999; Langdahl et al. 1998; Uitterlinden et al. 1998).

No association was seen between genotype and adjusted BMD at either LS ($p = 0.8$) or FN ($p = 0.8$) in the population as a whole. Addition of height, weight and gender as covariates did not alter this result.

However, when split into tertiles according to age, a significant association was seen between COL1A1 genotype and BMD in the oldest tertile (age range 65.7 to 90.4 years) at LS ($p=0.02$) with a similar trend evident at FN ($p=0.08$). In contrast with previous studies, BMD was lowest in SS homozygotes, the genotype previously associated with higher BMD (see Diagrams 6.2.5.1 and 6.2.5.2).

Diagram 6.2.5.1:
LS BMD in age tertiles according to COL1A1 genotype

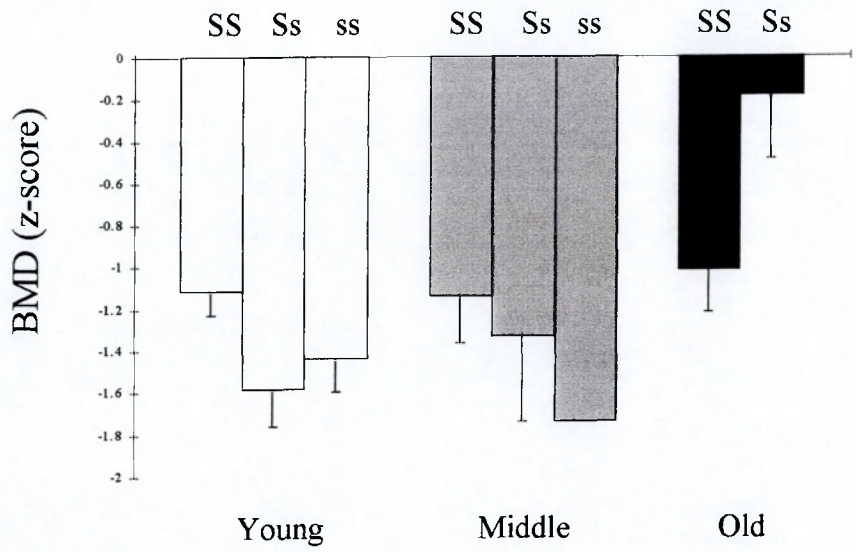
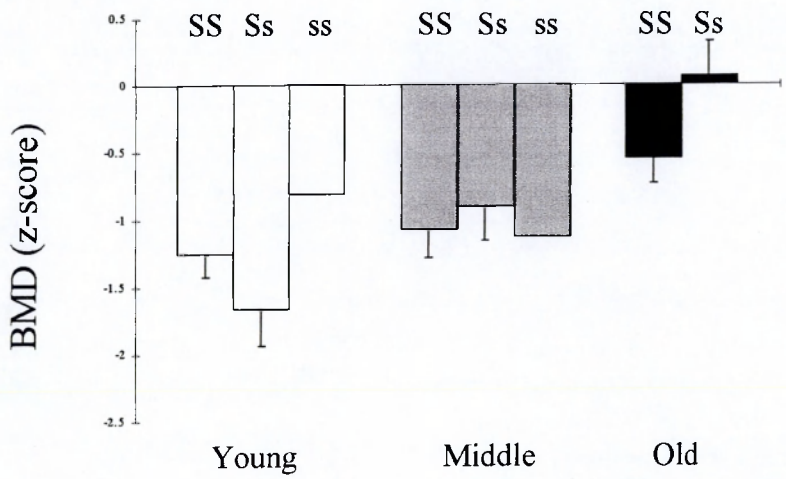


Diagram 6.2.5.2:
FN BMD in age tertiles according to COL1A1 genotype



6.2.5 Discussion

The results of this study conflict with several previous association studies of COL1A1 genotype and BMD. There are several potential explanations for this discrepancy.

The individuals used were selected from the large family cohort recruited through probands with an extreme BMD trait. The probands may have had low BMD due to either low peak bone mass or due to excessive bone loss. The mean age of the probands was 53.4 years in male probands and 51.4 years in female probands, and BMD was significantly low in the young relatives (presented in Chapter 3). This suggests that in this cohort the genetic effect upon BMD is largely due to an effect upon low peak bone mass.

Several previous studies have suggested that the COL1A1 polymorphism might affect bone loss rather than peak bone mass. Uitterlinden and colleagues (Uitterlinden et al. 1998) showed association of the polymorphism with BMD at both femoral neck and lumbar spine, with evidence of a gene-dose effect of the 's' allele in lowering BMD. However, the differences in BMD for each genotype became greater with age, with no significant difference between genotypes evident in younger women, consistent with the polymorphism determining rates of bone loss. A cross-sectional study supported a role for the polymorphism in determining rates of bone resorption, with increased levels of pyridinoline crosslinks (though not deoxypyridinoline) in women possessing the 's' allele (Keen et al. 1999). In a longitudinal study, although no difference in BMD between COL1A1 genotypes was observed at baseline, there was greater loss of total BMD in the 'ss' genotype group in both men and women compared with the other two genotypes. A

similar but non-significant trend was evident at femoral neck but not at lumbar spine (Harris et al. 2000). Thus the lack of an association in this study may be due to a cohort selected particularly for heritability of low peak bone mass.

Consistent with a lack of effect of COL1A1 alleles upon peak bone mass, a small twin study in young women (mean age 33.5 years) did not show any evidence of linkage or association at this locus (Hustmyer et al. 1999). This may be due to type 2 error as the sample size in this study was very small. There was no association of the Sp1 polymorphism with forearm, lumbar spine or total body BMD in a study of children and adolescents (Berg et al. 2000). However, another group has found an association between density of cancellous bone at lumbar spine measured by QCT and carriage of the 's' genotype in a group of prepubertal girls (Sainz et al. 1999).

Almost all previous studies of the COL1A1 polymorphism and BMD have been in women only. The current study used both men and women. Despite adding gender in as a covariate, a gender-specific effect on BMD in women only may have been missed due to the small numbers in the female group.

Gene-environment interaction could also contribute to these results. Strong evidence of such interaction was reported in a longitudinal study of the effect of COL1A1 upon bone loss and its interaction with calcium intake ($p=0.0006$) (Brown et al. 2001). The "at risk" 's' allele was associated with bone loss at the lumbar spine (but not at femoral neck) in elderly postmenopausal females only in those with low dietary calcium intake ($p=0.01$). The opposite effect was present in those on a high dietary calcium intake, such that 's' carriers gained bone relative to 'SS' homozygotes ($p=0.003$). Thus the effect of the

COL1A1 genotype on BMD may be substantially affected by environmental influences such as calcium intake, which vary widely across different Caucasian groups (Brown et al. 2001).

Alternatively, the results of this study could be explained by different linkage disequilibrium between the true osteoporosis-inducing polymorphism and alleles of the Sp1 site in different populations. Linkage disequilibrium between the Sp1 polymorphism site and a deletion in the upstream regulatory region of the COL1A1 gene has been identified in a Spanish population (Nogues et al. 2000), although no association of the deletion with BMD was observed. A further polymorphism in the upstream regulatory region of the COL1A1 gene identified in a Spanish population has been associated with BMD (Garcia-Giralt et al. 2000). However, McGuigan and colleagues (McGuigan et al. 2000) have argued that it is allelic variation at the Sp1 site, rather than at other close by sites, that affects fracture susceptibility. Although there was substantial linkage disequilibrium between several polymorphims of COL1A1 and its surrounding region, the Sp1 polymorphism was found to be the only independent predictor of fracture (McGuigan et al. 2000). However, this does not necessarily mean that variability at the Sp1 site is responsible for determining fracture risk (although this may be the case); rather that linkage disequilibrium between the true disease-causing polymorphism and the Sp1 site is greater than with any of the other polymorphic regions studied. Of note, the Sp1 polymorphism site is not found in all populations (Han et al. 1999). The observed low BMD in the 'SS' genotype evident in the eldest tertile could occur due to linkage disequilibrium with a gene determining bone loss and the 'S' allele in the Oxford population.

Not all cross-sectional and longitudinal studies of the polymorphism have supported association with BMD, nor with markers of bone formation or resorption (Garnero et al. 1998; Heegaard et al. 2000; Hustmyer et al. 1999; Liden et al. 1998). However, as was the case for many association studies of VDR, studies with inadequate sample size will lack sufficient power to reject the null hypothesis. The power of the current study was also low.

The power of a study is the probability of not making a type 2 error, i.e. the probability of being able to correctly reject the null hypothesis. Power can be expressed as a percentage as 100 – b%, where b = probability of type 2 error. Power is affected by sample size so that larger samples result in greater power (as the sampling distribution curves narrow, there will be less overlap between the curves for cases and controls, making it more likely that a difference between the two will be detected). Additionally the magnitude of an effect (in this case, the effect of carriage of the mutant allele upon BMD) will affect power. The greater the effect of the polymorphism upon BMD, the greater the power of the study to detect the difference between genotypes. Assessing the size of an effect may be very difficult *a priori*, as if it were known then the study would usually be unnecessary. Thus power is often assessed either retrospectively or for estimates of the expected effect.

A negative association study may have power to exclude a polymorphism from having a major effect upon a phenotype but may have insufficient power to exclude a more subtle effect. The lack of association in this study may result from the low power of the study.

In summary, no association of alleles of COL1A1 and BMD was observed in this cohort as a whole. Although an effect was seen in the oldest tertile, it was with the opposite allele to that previously reported. This may have arisen due to differing genetic effects upon peak bone mass and bone loss, in a population primarily selected for genetic effects upon peak bone mass. Gene-environment interactions may result in different associations being seen with different populations. Alternatively linkage disequilibrium between the Sp1 polymorphism and the 'real' disease-causing gene may be different in different populations. However, the power of this study to exclude an association was low.

6.3 A Within-Family Association Study of the COL1A1 Polymorphism and BMD, using Quantitative Transmission Disequilibrium Statistics

Association of BMD with the COL1A1 Sp1 polymorphism was sought using quantitative TDT in a cohort of families selected through probands with osteoporosis. A discussion of within-family association methods including transmission disequilibrium statistics is presented in Chapter 1.

6.3.1 Participants

Probands and families were recruited as described in Chapter 3.

6.3.2 Genotyping

Genotyping for the COL1A1 was performed as described in section 6.1.3.

6.2.4 Statistical Methods

The families were analysed as general pedigrees (i.e. the pedigrees were not broken into nuclear families). Identity-by-descent (IBD) parameters were obtained by using the programme SimIBD (Sobel et al. 1996). SimIBD uses descent vector pathway analysis to determine IBD status, using multiple simulations to determine the most likely pathway.

The IBD data was then analysed with the pedigree, genotype and phenotype information in the QTDT programme (Abecasis et al. 2000). QTDT measures association using an orthogonal general model of association, in which total association is split into between and within family components. Additionally variance components modelling is used to partition total variance into its components (including variance from shared and non-shared environment, and genetic variance, due to additive and dominance effects at a locus with a residual polygenic component). For association and linkage, the null model (incorporating the mean trait value, important covariates, and between-family variance) is compared with the full model (incorporating within-family variance in addition to the other factors), and statistical significance is assessed by the log-likelihood test. The power to detect association is greater than the power to assess linkage. Linkage and association can also be assessed in isolation. Parent-of-origin effects can be included in the model. The programme also allows for departure from multivariate normality, through the use of Monte Carlo permutation tests to determine statistical significance.

Overall association of the Sp1 polymorphism with BMD at both LS and FN was assessed. Additionally, maternal and paternal transmissions were compared for a parent-

of-origin effect upon the trait (i.e. imprinting). Evidence for association independent of linkage, and of linkage independent of association, was also assessed.

The cohort was assessed for population stratification.

The polymorphism information content (PIC) of the COL1A1 Sp1 polymorphism was calculated according to the formula given in Section 2.3.1, using the programme Downfreq (Lathrop et al. 1984).

6.2.5 Results

Overall, neither linkage nor association was seen at either femoral neck or lumbar spine ($p>0.5$ for both), assessable using 480 individuals. However, a parent-of-origin effect was evident at FN ($p=0.019$) but not at LS. This was assessable using 72 probands. Additional analysis of association only supported a parent-of-origin effect (using 480 individuals). The parent-of-origin effect was entirely due to maternal transmissions, with a maternal effect on allele transmission upon BMD at FN ($p=0.0078$). For this calculation 33 transmissions were used, as this was only assessable from heterozygous mothers. Paternal transmissions did not have a significant effect at either site, with 39 transmissions assessable.

Use of the Monte Carlo permutations test still demonstrated a significant parent-of-origin effect upon femoral neck BMD ($p=0.008$), due entirely to maternal effect ($p=0.012$).

There was no evidence of linkage independent of association, nor or association independent of linkage.

The effect of maternal transmission of the 'S' allele resulted in an increase in BMD of 0.126 SD, whereas maternal transmission of the 's' allele resulted in a decrease of BMD, also of 0.126 SD.

The PIC of this polymorphism was low (0.237), making it a poor marker for linkage (Camp 1997; Risch 1990).

No population stratification was evident. Thus it was reasonable to use this cohort for unrelated population association studies.

6.3.5 Discussion

Consistent with the negative association demonstrated in the population association study of BMD and COL1A1 genotypes, overall no linkage or association was evident at either femoral neck or lumbar spine using transmission disequilibrium statistics.

This was unexpected, given the previous demonstration of linkage of this locus with BMD at femoral neck (LOD=1.7) (presented in Chapter 4). TDT is a measure of both linkage and linkage disequilibrium, but in the absence of linkage disequilibrium the test will fail to detect linkage as well. Neither linkage nor overall association were evident when each was examined independently.

The lack of evidence of linkage may be due to the very low informativity of this marker. Not only is it biallelic, one allele is rare, resulting in many uninformative meioses. As discussed in Section 2.3.1, the power of linkage is directly related to the informativity of the markers used.

A significant parent-of-origin effect was evident at femoral neck, due entirely to a maternal effect. This is consistent with the genetic epidemiology results presented in Chapter 3, showing gender- and site-specificity of inheritance of BMD.

Parent-of-origin effects imply imprinting. Imprinting is an epigenetic phenomenon specific to mammals. Parental origin is 'marked' upon chromosomes independently of the DNA sequence, resulting in expression of imprinted genes according to their parental origin. Although two alleles are nominally inherited from the parents, only one is transcriptionally active, with the other silenced by methylation. For example, IGF-II expression in mice occurs solely from the paternally derived allele whereas IGF-II receptor gene expression occurs solely from the maternal allele. In humans, imprinting is exemplified by the differing genetic defects resulting from loss of chromosome 15q11-13. Loss of the paternal allele results in Prader-Willi syndrome (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?176270), with disease manifest by diminished foetal activity, obesity, type 2 diabetes mellitus, mild mental retardation, hypotonia, hypogonadotrophic hypogonadism, short stature, and small hands and feet. Loss of the maternal allele results in Angelman syndrome with severe intellectual and motor retardation, hypotonia, lack of speech, inappropriate laughter, and

tongue thrusting (previously descriptively referred to as the 'happy puppet' syndrome) (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?105830).

Imprinting occurs during formation of germ cells. During early development of the blastocyst, a wave of demethylation sweeps over the entire genome, followed by a wave of remethylation. Imprinted genes, however, are resistant to these waves of demethylation and remethylation, retaining the pattern set according to their parent of origin. Primordial germ cells, however, do not undergo remethylation. During germ cell development and gametogenesis, *de novo* methylation occurs, setting a new, sex-specific methylation pattern. This *de novo* pattern will be carried in the haploid genome of the male and female gametes, imprinting the parental origin of the chromosomes. Whether methylation is the primary cause of imprinting or whether it is secondary to sex-specific chromatin modifications is uncertain.

The spectrum of action of imprinted genes is still being determined, although many imprinted genes are involved in foetal growth. The rationale for imprinting is not known, however a theory of 'parent-conflict' proposes paternally imprinted genes favour growth of the foetus and placenta at the expense of the mother, whilst maternal imprinted genes reduce growth rates of the foetus (reviewed by (Jaenisch 1997), with further discussion at http://www.geneimprint.com). *In utero* effects upon bone development and growth may effect subsequent peak bone mass. However, as discussed above, the evidence to date is that the effect of this polymorphism of COL1A1 may be greatest for bone loss.

Evidence of site-specific effects in osteoporosis was initially suggested by Seeman and colleagues, who demonstrated that premenopausal daughters of osteoporotic mothers

with lumbar spine fracture have lower BMD at the spine than at other sites (Seeman et al. 1989). Further, daughters of mothers with femoral neck fractures only had low BMD at the femur (Seeman et al. 1994). Site-specificity of inheritance of fracture risk also supports this concept (Fox et al. 1998). The genetic epidemiology on this cohort of families presented in Chapter 3 also suggested site-specific effects, with evidence of dominance variance at the FN but not LS, and gender-specific effects upon BMD at different sites. Demonstration of a maternal effect upon BMD at the femoral neck is entirely consistent with these epidemiological results.

The maternal transmission of the 's' or 'S' allele significantly affected femoral neck BMD to the extent of decreasing or increasing BMD by approximately 0.126 SD, accounting for 15% of overall variance of BMD at FN. Thus this polymorphism only contributes a small amount to the overall heritability of BMD at FN.

Variance components analysis may not be robust if there is non-normal distribution of phenotype, as was the case with proband recruitment for the family collection. However, use of the Monte Carlo permutation test still showed a positive result for parent-of-origin effects due to maternal transmissions. This indicates that the positive results obtained were not due to the ascertainment bias inherent in our population (with its violation of multivariate normality).

In summary, a maternal affect upon the association of COL1A1 upon BMD was demonstrated at the femoral neck but not at the lumbar spine. This may be due to imprinting at the COL1A1 locus, contributing to site- and gender-specific effects upon the heritability of BMD.

Chapter 7: Association Studies of PTHR1

7.1 Introduction

The linkage study presented in Chapter 4 identified linkage of PTHR1 with BMD. However, linkage studies do not identify association of a particular allele with the trait in question. Within each family, linkage disequilibrium of the marker allele and the trait-determining locus allele is needed for linkage to be detected – however, co-segregation of these particular alleles may be unique to each family. Further, linkage studies are poor at localising the allelic variants underlying the observed linkage.

In this chapter association studies of the PTHR1 locus are presented, using the M7 polymorphism previously identified in this population (Chapter 5) and by others (Hustmyer et al. 1993; Schipani et al. 1994). The use of SNPs in mapping studies is based upon the likelihood of linkage disequilibrium between such polymorphisms and the disease-causing allele. The M7 polymorphism does not result in a coding change or splice site, and therefore is unlikely to be the disease-causing mutation itself. However, it may be in linkage disequilibrium with the polymorphism(s) of the PTHR1 locus contributing to the variance of BMD.

A restriction fragment length polymorphism (RFLP) assay was designed for this chapter. RFLP assays provide a rapid and inexpensive way of genotyping. Use of restriction enzymes also corroborates the accuracy of polymorphism detection by sequencing, as their action is critically dependent upon the underlying base sequence.

Three separate association studies are presented in this study. Firstly, a small case-control study was performed using unrelated individuals selected from the family collection for high and low bone density (see Chapter 3 for details of family recruitment). Secondly, the whole family collection was used for TDT analysis. Finally, a large population based association study was undertaken at Erasmus University, Rotterdam, The Netherlands, using participants from The Rotterdam Study.

7.2 An Association Study of a Polymorphism of PTHR1 in Individuals with Extremes of BMD

7.2.1 Methods

7.2.1.1 Population studied

72 unrelated individuals were selected from the family collection described in Chapter 3. 36 samples from individuals with low BMD were previously used in PTHR1 polymorphism screening (see Chapter 5). 36 unrelated individuals with the highest BMD in the cohort were chosen for study. Equal numbers were chosen to give greatest power per unit of genotype.

Mean femoral neck z-score in the low BMD group was –2.45 (range –3.31 to –1.96). 23 samples were from women, 13 from men.

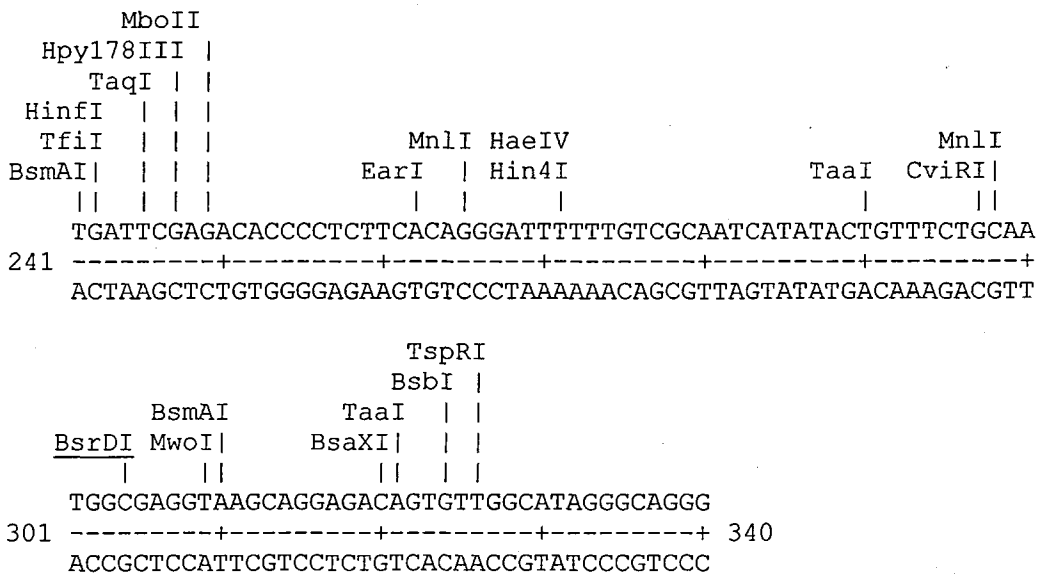
Mean femoral neck z-score in the high BMD group was 1.23 (range 0.55 to 2.74). 16 samples were from women, 20 were from men.

7.2.1.2 Laboratory Methods

The programme GCG (Genetics Computer Group Inc. (also known as Wisconsin Package), Oxford Molecular group Inc., http://www.gcg.com) was used to determine possible restriction enzymes for the M7 polymorphism found in Chapter 5 (see Diagram 7.2.1).

Diagram 7.2.1: Restriction Enzyme Cutting Sites for Exons M6-7 Polymorphism

(Bases 241 – 340 shown here as position of polymorphism is bp 301)



The restriction enzyme *Bsr DI* (New England Biolabs (UK) Ltd, Hitchin, Hertfordshire, UK) was chosen for this assay. This enzyme (underlined in Diagram 7.2.1) cleaves the mutant 'T' allele (see Diagram 7.2.2).

Diagram 7.2.2: Base sequence recognised by *Bsr DI*

Points of cleavage are marked by triangles.

Polymorphic bases are underlined.

5' – GCAATGNN▼ – 3'

3' – CGTTAC▲NN – 5'

The original M6-7 product was 340 bp long, which resulted in cleaved products of 302/304 and 36/38 bp. New primers were designed to shift the position of the polymorphism more centrally within the PCR product, in order to improve recognition of different genotypes. The new product was 256 bp, which when cleaved resulted in products of 162/164 and 92/94 bp.

PCR mix for M6-7 was 20ng DNA, 0.4μL of 5mM primers (forward and reverse), 1μL PCR buffer, 1.5mM $MgCl_2$, 2 units DNA Polymerase (either Bioline Taq (Bioline UK Ltd, London, UK) or Expand™ High Fidelity PCR enzyme (Boehringer Mannheim, Lewes, UK), 0.25μL of 2mM dNTPs and SDW to 10μL final volume. The forward primer was GGCAAGTCCAGATGCACTATG. The reverse primer was CAGGGTGGAAGAATGGAG. The PCR protocol consisted of 94°C for 2 minutes, followed by 32 cycles of ([94°C for 1 minute], [55°C for 1 minute], [72°C for 45 seconds]) using MJ thermal cyclers (MJ Research, Watertown, MA, USA). PCR was finished with 10 minutes at 72°C. Post PCR, 10μL of SDW was added to the product to dilute it 1 in 2.

The reaction mix for RFLP consisted of 3μL of diluted PCR product, 1μL enzyme (= 2 units/reaction), 0.1μL BSA, 1μL enzyme buffer with SDW to 10μL volume. This was incubated for a minimum of an hour at 60°C before running out on a 3% agarose gel. Of note, this reaction was still complete using as little as 0.25μL of enzyme (= 0.5 units).

7.2.1.3 Statistical Methods

Allele frequencies were calculated from the observed genotype frequencies obtained using the programme Genotype Relative Risk (Lathrop 1983). Genotypes were compared using a 3x2 contingency table and the χ^2 statistic. Fisher's Exact test was used to compare allele frequencies and carriage of the mutant allele compared with noncarriage, using the LINKAGE statistical package (Lathrop et al. 1984). Hardy-Wienberg equilibrium was assessed using the Genotype Relative Risk programme (Lathrop 1983).

7.2.2 Results

A total of 70 genotypes were unequivocally identified. Genotype frequencies and their comparison between high and low BMD groups are presented in Table 7.2.2.1. Allele frequencies and comparisons are presented in Table 7.2.2.2. Both high and low BMD genotypes were in Hardy-Weinberg equilibrium.

Table 7.2.2.1: Genotype Frequencies for M7 Polymorphism in Extreme BMD Groups

Genotype	Low BMD Number (frequency)	High BMD Number (frequency)
Wild-type homozygotes	10 (0.28)	19 (0.54)
Heterozygotes	22 (0.63)	14 (0.41)
Mutant homozygotes	3 (0.09)	2 (0.06)
Total	35	35

$\chi^2 = 4.25$, $p = 0.12$.

Table 7.2.2.2: Alleles for M7 Polymorphism in Extreme BMD Groups

Allele	Low BMD Number (frequency)	High BMD Number (frequency)
Wild-type	42 (0.6)	52 (0.74)
Mutant	28 (0.4)	18 (0.26)
Total	70	70

Comparison of allele numbers using 2x2 Contingency table – $\chi^2 = 2.84$, $p = 0.18$.

A non-significant increase in carriage of the mutant allele in the low BMD group was observed, although the main contribution to this finding was an overrepresentation of heterozygotes. Therefore, the genotypes were grouped and carriage of the mutant allele (homozygous mutants and heterozygous genotypes) was compared with non-carriage of the mutant allele (homozygous wild-type) with observed low BMD genotypes compared with 'control' high BMD genotypes calculated according to Hardy-Weinberg expectations.

Table 7.2.2.3: Carriage of M7 mutant allele in individuals with low BMD

	Numbers (Observed Frequency)	**Numbers (Expected Frequency)**
Carriage of mutant allele	25 (0.71)	15.75 (0.45)
Non-carriage of mutant allele	10 (0.29)	19.25 (0.55)

Using Fisher's exact test, p = 0.034 (two-sided).

7.3 A Within-Family Association Study of M7 Polymorphism in the PTHR1 gene and BMD, using Quantitative Transmission Disequilibrium Statistics

7.3.1 Methods

7.3.1.1 Population Studied

Probands and families were recruited as described in Chapter 3.

7.3.1.2 Laboratory Work

Genotyping of the M7 polymorphism was performed as described above.

7.3.1.3 Statistical Methods

Quantitative transmission disequilibrium analysis of the M7 polymorphism was performed as described in Section 6.2.3. BMD was analysed at both LS and FN. Height, weight, and age were used as covariates. Overall association of the M7 polymorphism with BMD at both LS and FN was assessed. Additional analyses included assessment of

parent-of-origin effect upon the trait (i.e. imprinting). Evidence for association independent of linkage and of linkage independent of association was also assessed.

The polymorphism information content (PIC) of the M7 polymorphism was calculated according to the formula given in Section 2.3.1, using the programme Downfreq (Lathrop et al. 1984).

7.3.2 Results

A significant association of the M7 polymorphism was seen with BMD at LS ($p = 0.04$) but not at FN. Transmission of the mutant allele resulted in a decrease of 0.1 SD, whereas transmission of the wild-type allele resulted in an increase of 0.1 SD. This accounted for approximately 15% of the total variance of the trait. Use of the Monte Carlo permutation tests supported the significance of this result.

There were insufficient informative transmissions for assessment of parent-of-origin effects. No significant linkage was observed with this marker.

The PIC of the marker was 0.2.

7.4 A Population-Based Association Study of the M7 Polymorphism in the PTHR1 gene

This work was carried out in Dr Andre Uitterlinden's laboratory, Department of Internal Medicine, Erasmus University, Rotterdam, The Netherlands.

7.4.1 Methods

7.4.1.2 Population Studied

The population recruited for the Rotterdam Study has been previously described (Uitterlinden et al. 1998). In brief it is a population based cohort study of men and women aged 55 and older, recruited from the Ommoord district of Rotterdam, The Netherlands. BMD data and DNA samples were available from a total of 1814 individuals, all of which were used in this study.

7.4.1.3 Laboratory Methods

PCR was reoptimised to adjust for different concentrations and purity of the DNA. The new reaction mix consisted of 1μL of DNA stock solution (approximately 100ng of DNA per reaction), 1mM $MgCl_2$, 0.125μL of 2mM dNTPs, 0.4μL of 5mM primers, 1μL PCR buffer and 0.1μL Bioline Taq (Bioline UK Ltd, London, UK). Further laboratory methods were as described above.

7.4.1.4 Statistical Methods

BMD at LS and FN was compared between the three genotype groups using analysis of covariance (described in Section 6.1.4). Multiple linear regression was used to adjust BMD for relevent clinical covariates including BMI, gender and age. The cohort was stratified into tertiles according to age, in order to assess for any changing effects of PTHR1 genotype on BMD and fracture risk with age.

7.4.2 Results

BMD and genotyping data was available for a total of 1788 people (912 men, 876 women). Genotype frequencies were in Hardy-Weinberg equilibrium for the total group, for each gender group and within each age tertile. The age tertiles are shown in Table 7.4.2.1.

Table 7.4.2.1 Age Tertiles for Participants in the Rotterdam Study (in years)

Tertile	Males	Females
1	55.18-63.46	55.01-63.28
2	63.47-71.48	63.29-71.5
3	71.49-79.92	71.51-79.98

A significant association of lower BMD (in g/cm^2 (unadjusted for age or gender) and as z-scores) with the 'C' polymorphism was noted at LS in women in the oldest age tertile (Table 7.4.2.5 and 7.4.2.9). A non-significant trend for this association was also observed in men overall, at LS ($p = 0.132$ with ANOVA and 0.07 after multiple regression) and FN (although with much smaller differences in BMD between genotypes, $p = 0.56$) (Table 7.4.2.6). This trend was evident in the second and third age tertiles for men (Tables 7.4.2.4-5 and 7.4.2.8-9). Of note, a non-significant trend for the opposite association was noted with women in the second age tertile ($p = 0.16$ with ANOVA and 0.06 after multiple regression) (Table 7.4.2.4 and 7.4.2.8).

Inclusion of BMI as a covariate increased the significance of association of the 'C' allele with low BMD (in g/cm^2) at LS in women in the oldest tertile ($p = 0.008$) (Table 7.4.2.13). The non-significant trend for the same association in men in the second and

third age tertiles at both FN and LS remained (Tables 7.4.2.12-13). Non-significant trends were also seen for an effect of this allele upon BMD at LS in women in the second age tertile (Table 7.4.2.12) and in men at FN in the first age tertile (Table 7.4.2.11), though in the opposite direction.

Tables 7.4.2.2: BMD in g/cm^2 (mean ± SD) of FN and LS by PTHR1 genotype

PTHR1	Men			Women		
	n	FN	LS	n	FN	LS
TT	345	0.877 ± 0.132	1.169 ± 0.191	355	0.806 ± 0.127	1.019 ± 0.173
TC	435	0.868 ± 0.134	1.144 ± 0.194	386	0.798 ± 0.119	1.003 ± 0.164
CC	132	0.862 ± 0.127	1.142 ± 0.199	135	0.803 ± 0.129	1.031 ± 0.170
P-value: ANOVA		0.45	0.15		0.69	0.19
P-value: regression			0.08			

Tables 7.4.2.3-5: BMD in g/cm^2 (mean ± SD) of FN and LS by PTHR1 genotype, stratified by age tertiles

Table 7.4.2.3 Age tertile 1	Men			Women		
Genotype	**n**	**FN**	**LS**	**n**	**FN**	**LS**
TT	119	0.903 ± 0.110	1.177 ± 0.167	120	0.845 ± 0.125	1.041 ± 0.155
TC	148	0.888 ± 0.120	1.139 ± 0.168	125	0.829 ± 0.120	1.017 ± 0.157
CC	36	0.926 ± 0.144	1.173 ± 0.207	48	0.844 ± 0.105	1.078 ± 0.175
P-value: ANOVA		0.20	0.17		0.55	0.08
P-value: regression						

Table 7.4.2.4 Age tertile 2	Men			Women		
	n	**FN**	**LS**	**n**	**FN**	**LS**
TT	121	0.859 ± 0.144	1.141 ± 0.196	124	0.808 ± 0.127	0.987 ± 0.173
TC	132	0.859 ± 0.141	1.117 ± 0.194	124	0.804 ± 0.115	1.019 ± 0.161
CC	51	0.844 ± 0.109	1.109 ± 0.200	44	0.809 ± 0.140	1.036 ± 0.169
P-value: ANOVA		0.77	0.50		0.97	0.16
P-value: regression						0.06

Table 7.4.2.5 Age tertile 3	Men			Women		
	n	**FN**	**LS**	**n**	**FN**	**LS**
TT	105	0.868 ± 0.136	1.193 ± 0.208	111	0.762 ± 0.117	1.031 ± 0.185
TC	155	0.856 ± 0.138	1.172 ± 0.215	137	0.765 ± 0.113	0.976 ± 0.169
CC	45	0.832 ± 0.116	1.154 ± 0.189	43	0.750 ± 0.124	0.974 ± 0.152
P-value: ANOVA		0.31	0.53		0.78	0.03
P-value: regression						0.02

Table 7.4.2.6: BMD in z-scores (mean ± SD) at FN and LS by PTHR1 genotype

All ages	Men			Women		
	n	FN	LS	n	FN	LS
TT	345	-0.005 ± 0.965	0.027 ± 0.992	355	-0.064 ± 0.945	-0.098 ± 0.954
TC	435	-0.067 ± 0.981	-0.104 ± 1.009	386	-0.111 ± 0.899	-0.184 ± 0.902
CC	132	-0.098 ± 1.009	-0.118 ± 0.926	135	-0.077 ± 0.942	-0.028 ± 0.928
P-value: ANOVA		0.563	0.132		0.79	0.19
P-value: regression			0.07			

Table 7.4.2.7-9: BMD in z-scores (mean ± SD) at FN and LS by PTHR1 genotype, in age tertiles

Table 7.4.2.7 Age tertile 1	Men			Women		
	n	FN	LS	n	FN	LS
TT	119	0.009 ± 0.836	0.122 ± 0.845	120	-0.044 ± 0.958	-0.027 ± 0.858
TC	148	-0.109 ± 0.906	-0.069 ± 0.851	125	-0.168 ± 0.934	-0.161 ± 0.869
CC	36	0.179 ± 1.083	0.105 ± 1.052	48	-0.030 ± 0.790	0.181 ± 0.962
P-value: ANOVA		0.20	0.18		0.50	0.07
P-value: regression						

Table 7.4.2.8 Age tertile 2	Men			Women		
	n	**FN**	**LS**	**n**	**FN**	**LS**
TT	121	-0.139 ± 1.0874	-0.117 ± 0.991	124	-0.038 ± 0.974	-0.275 ± 0.959
TC	132	-0.138 ± 1.0715	-0.238 ± 0.980	124	-0.070 ± 0.879	-0.099 ± 0.891
CC	51	-0.256 ± 0.815	-0.280 ± 1.013	44	-0.030 ± 1.098	-0.000 ± 0.935
P-value: ANOVA		0.76	0.50		0.95	0.16
P-value: regression						0.06

Table 7.4.2.9 Age tertile 3	Men			Women		
	n	**FN**	**LS**	**n**	**FN**	**LS**
TT	105	0.134 ± 1.027	0.085 ± 1.049	111	-0.115 ± 0.903	0.023 ± 1.025
TC	155	0.034 ± 1.046	-0.024 ± 1.088	137	-0.095 ± 0.887	-0.281 ± 0.938
CC	45	-0.142 ± 0.880	-0.112 ± 0.956	43	-0.179 ± 0.940	-0.289 ± 0.836
P-value: ANOVA		0.31	0.53		0.87	0.03
P-value: regression						0.02

Table 7.4.2.10: BMD in g/cm^2 (mean ± SD), adjusted for BMI, at FN and LS by PTHR1 genotype

All ages	Men			Women		
	n	FN	LS	n	FN	LS
TT	344	0.873 ± 0.129	1.164 ± 0.185	353	0.807 ± 0.113	1.021 ± 0.169
TC	431	0.870 ± 0.125	1.148 ± 0.187	383	0.799 ± 0.117	1.005 ± 0.157
CC	130	0.867 ± 0.125	1.151 ± 0.182	134	0.801 ± 0.116	1.028 ± 0.162
P-value: ANOVA						
P-value: regression		0.88	0.50		0.66	0.28

Table 7.4.2.11-13 BMD in g/cm^2 (mean ± SD), adjusted for BMI, at FN and LS by PTHR1 genotype, in age tertiles

Table 7.4.2.11 Age tertile 1	Men			Women		
	n	FN	LS	n	FN	LS
TT	118	0.893 ± 0.119	1.167 ± 0.174	120	0.845 ± 0.109	1.042 ± 0.153
TC	147	0.896 ± 0.109	1.148 ± 0.170	123	0.831 ± 0.111	1.022 ± 0.155
CC	36	0.931 ± 0.114	1.178 ± 0.168	48	0.839 ± 0.118	1.074 ± 0.159
P-value: ANOVA						
P-value: regression		0.21	0.51		0.65	0.144

Table 7.4.2.12 Age tertile 2	Men			Women		
	n	FN	LS	n	FN	LS
TT	121	0.857 ± 0.132	1.137 ± 0.187	124	0.807 ± 0.122	0.985 ± 0.167
TC	131	0.857 ± 0.137	1.120 ± 0.195	124	0.805 ± 0.122	1.020 ± 0.167
CC	49	0.845 ± 0.133	1.118 ± 0.189	44	0.809 ± 0.120	1.037 ± 0.166
P-value: ANOVA						
P-value: regression		0.85	0.73		0.98	0.110

Table 7.4.2.13 Age tertile 3	Men			Women		
	n	FN	LS	n	FN	LS
TT	105	0.868 ± 0.133	1.193 ± 0.205	109	0.767 ± 0.104	1.037 ± 0.167
TC	153	0.855 ± 0.123	1.172 ± 0.198	136	0.766 ± 0.105	0.978 ± 0.163
CC	45	0.838 ± 0.127	1.163 ± 0.201	42	0.746 ± 0.110	0.964 ± 0.168
P-value: ANOVA						
P-value: regression		0.42	0.61		0.53	0.008

7.5 Discussion

The two association studies from the Oxford cohort demonstrate that carriage of the M7 polymorphism is associated with lower BMD. Although the results in the Dutch population were non-significant, the same trend was observed.

The first study compared femoral neck BMD between cases (low BMD) and 'hypernormals' (high BMD), as the use of 'hypernormals' as controls in a case-control association study is a more efficient design than is the use of 'normal' controls (Morton et al. 1998). Carriage of the mutant allele was greater in the low BMD group.

The M7 polymorphism was associated with BMD at LS in the QTDT analysis, but was not significantly associated with BMD at femoral neck. Linkage of PTHR1 had been observed with BMD at both LS and FN.

The proportion of total BMD variance explained by the M7 polymorphism was approximately 15%. This is equivalent to the effect on BMD of the COL1A1 Sp1 polymorphism (presented in Section 6.2.5).

A significant association of the M7 polymorphism with BMD was also demonstrated in the Rotterdam cohort. Carriage of the 'C' allele was associated with low BMD at LS in women in the most elderly tertile, with strengthening of association when BMI was included as a covariate. The same – although non-significant – trend was evident in men. This association of polymorphisms of M7 with BMD was in the same direction as that demonstrated for the Oxford cohort. It is also interesting to note that the effect was mainly seen at LS in the Rotterdam cohort, as it was for the Oxford cohort also.

The Rotterdam cohort is quite elderly in comparison to the Oxford families, with mean age of 70 years. Thus genetic effects upon bone loss rather than peak bone mass may contribute more to the overall heritability of BMD in this cohort. Indeed this has been previously argued with respect to this population. Association of polymorphisms of COL1A1 with BMD was only evident in the most elderly tertile of this population (Uitterlinden et al. 1998). The restriction of a significant association to the most elderly tertile of women suggests that this polymorphism has at most a small effect upon bone loss induced by menopause. The variability introduced by individual rates of menopausal bone loss may have masked the effects of this polymorphism upon BMD in the younger female tertiles. The underlying effect of PTHR1 polymorphism upon peak BMD, and/or upon bone loss associated with ageing, would only be evident after menopausal bone loss had ceased. The non-significant trend for the same association in men overall is consistent with an effect of this polymorphism upon peak bone mass, and/or bone loss associated with ageing.

Gene-environment interaction was analysed using the covariates of height, weight, BMI, age and gender. Other important environmental variates, such as calcium intake and estrogen exposure, have not been examined, and may have a significant effect upon the results obtained.

Although the M7 polymorphism is non-coding and probably has no functional effect, this result suggests that it is in linkage disequilibrium with a polymorphism within PTHR1 determining BMD. Whilst the extent of linkage disequilibrium in the general population is arguable (see Section 1.5.2), linkage disequilibrium with the M7 polymorphism would

not extend beyond the region of the PTHR1 gene, as the PTHR1 gene is quite large (20kB in coding region alone), significantly greater than even generous estimates of genome-average linkage disequilibrium. Further, the observed association is in an area already identified by linkage and in a candidate gene selected *a priori* in the region. Thus this association is unlikely to be a false positive result. Determination of the exact polymorphism of PTHR1 responsible for the variance explained at this locus is the subject of further work.

As discussed in Section 4.1, very few disease-causing genes have been identified in any complex genetic disease, including osteoporosis. This work has demonstrated both linkage and association of PTHR1 with BMD. Genetic variation at the PTHR1 locus contributes to the total heritability of BMD and to the development of osteoporosis.

Chapter 8: Future Directions

This thesis has demonstrated linkage and association of PTHR1 with BMD. Several other genes influencing BMD were also identified by linkage (COL1A1, VDR, ER-α, EGF, IL-1, IL-4, IL-6, RANKL) and association (COL1A1), with possible imprinting of the COL1A1 locus. A sibling recurrence risk ratio for low BMD has been established at LS and FN. Genetic epidemiological studies have suggested both site- and gender-specific effects upon BMD.

The exact polymorphisms of PTHR1 underlying population variability in BMD remain unidentified. Although association was detected with the M7 exon polymorphism and low BMD, this polymorphism is non-coding. Thus it is likely that the M7 exon polymorphism is in linkage disequilibrium with a polymorphism affecting PTHR1 function and, in turn, BMD. A key issue therefore is the determination of the polymorphism truly associated with low BMD. The novel polymorphism identified in U3 is also of great interest, given its location within a critical region of the promoter region of PTHR1. The U4 exon remains to be assessed for polymorphic variation, with the more accurate sequence data generated from the Human Genome Project aiding this work.

One possible way of screening PTHR1 exons and promoter regions would be to use single nucleotide polymorphisms (SNP) identified in and around this gene (such data is freely available at http://snp.cshl.org/snp and http://www.ncbi.nlm.nih.gov/SNP). Of note, there is a relative paucity of non-conservative SNPs in coding regions, compared with either conservative or synonymous polymorphic variations (Cargill et al. 1999;

Halushka et al. 1999) as well as relative deficiency of allelic diversity in 5' untranslated regions (Halushka et al. 1999). This presumably has arisen because of selective pressure against the survival of such polymorphisms, as they would be expected to have deleterious functional effects upon the gene. Similarly, the heterozygosity of both non-conservative and conservative coding region SNPs is lower than for SNPs overall (Cargill et al. 1999; Halushka et al. 1999). This may affect the ability to detect association of PTHR1 with BMD using SNP mapping, in both within-family and population association studies.

Once a disease-associated polymorphism is identified, functional studies will be needed. Osteoblast-like or renal-derived cell lines could be transfected with different isoforms of PTHR1 to study the effect of receptor polymorphism upon response to PTH and PTHrP. PTH and/or PTHrP infusions in humans of different genotype may demonstrate differing effects upon cAMP, calcium and phosphate handling by calcitropic tissues such as kidney and bone.

Other candidate genes identified in this linkage study also need further mapping work to refine the areas of linkage and identify the disease-associated variants.

The linkage work undertaken in this study looked only at a limited number of candidate genes. Linkage results from candidate genes studies may have lower false-positive rates than those from a genome-wide scan. Candidate genes are chosen because of *a priori* evidence of their involvement in the trait under study, and thus the prior probability that a positive linkage result is a true positive is greater than for random microsatellite markers

spread across the genome. Nonetheless, a genome-wide scan might identify novel areas affecting BMD.

To date, linkage studies of whole genome scans utilising microsatellite markers have had limited success in definitive gene mapping in complex diseases such as osteoporosis. Risch recently compared the power of linkage studies (using the affected sib pair method) with association studies (using a population case-control approach) to detect disease-causing loci (Risch 2000). For loci with high genotype relative risk (>4) and intermediate allele frequencies (allele frequencies of 0.05-0.5) linkage studies should have sufficient power to locate disease loci, without requiring a prohibitive large sample. However, for lesser genotype relative risks (<2), linkage would fail to detect disease loci whereas a case-control association study would still have sufficient power to detect such genes (Risch 2000). BMD is a polygenic trait (as discussed in Chapter 1), and the effects upon BMD from each individual locus may be small. Therefore an alternative approach to whole genome linkage mapping may be appropriate. As has been mentioned, this could include SNP association studies, either within-family or at a population level. The extent of linkage disequilibrium in the population will critically determine the feasibility of such association studies. Differences in population linkage disequilibrium can be exploited to differentiate causal association from linkage disequilibrium. A population with high linkage disequilibrium could be used for initial screening to detect SNP associations; a population with low linkage disequilibrium could then be screened to assess such associations to pinpoint the exact causal polymorphism (Risch 2000).

A further question for future linkage work in osteoporosis is the type of family to collect. For this project, a number of families with complex pedigrees were recruited, especially as this proved an efficient means of recruitment. However, the complex pedigrees were extremely difficult to analyse as a whole unit, and therefore were broken into nuclear families, with a theoretical loss of power to detect linkage. As discussed in Chapter 4, it may not be valid to make comparisons of bone phenotype across generations. It may be appropriate to limit linkage analysis to an affected sib-pair method (such as is utilised in MapMaker/Sibs), using parents only to establish IBD status. In addition to simplifying analysis, other advantages of this approach are that it would require less phenotyping, a major expense in genetic studies of BMD, and that sib-pairs similar in age will have more homogeneous genetic and environmental influences upon their BMD. Ideally, families containing two siblings concordant for very low BMD, or two siblings highly discordant in BMD, are the most powerful for linkage analysis. Specific powerful statistics have been developed for linkage analysis using such dual ascertainment approaches (e.g. the EDAC statistic (Gu et al. 1997)). Alternatively, single ascertainment schemes (such as that employed in this project) can be analysed using standard ascertainment-corrected variance components methods with only minor loss of power, but considerably greater ease of recruitment, compared with dual ascertainment schemes.

The suggestion that there may be site- and gender-specific effects upon BMD raises further issues with respect to recruitment and protocol of genetic studies in BMD. If the main site of interest in osteoporotic studies is femoral neck, then the most powerful study design may be to include only probands recruited through femoral neck, with a similar plan for studies of osteoporosis at lumbar spine. Further, subdividing analysis according

to gender (for example, using either male probands and their male relatives or female probands and their female relatives) may show evidence of gender-specific effects at either site. This study had insufficient power for these analyses.

This study used BMD at femoral neck as its measure of osteoporosis at the hip. A future project will be to analyse the data using BMD at total hip, as this measurement has been shown to be more precise than femoral neck measurement. This study also used the normative database provided by Hologic manufacturers to define z-scores at both LS and FN. A normative locally-obtained database would be the most appropriate, however in its absence the NHANES normative database may provide an acceptible alternative for defining z-scores at the hip for future work. Measurement of BMD at LS using lateral scanning would also result in more precise measurement of BMD due to loss of confounding effects from artefact such as vascular calcification, fracture, and osteophytes. Use of CT scanning to gain true volumetric BMD of trabecular bone involves unacceptable levels of radiation exposure for a voluntary study. All these measures may improve power of both linkage and association studies, by increasing the precision of the phenotype under consideration.

The suggestion that ultrasound measurements may reflect important qualities of bone not considered in DXA scanning means that future genetic studies in osteoporosis may also have to consider this different modality of bone density measurement.

Information upon important environmental influences upon BMD was collected from pariticipants in this study. This was mainly used to exclude BMD z-scores from people with secondary osteoporosis. However, the residual information needs to be analysed

and considered for inclusion as covariates in analysing genetic effects upon BMD. In particular, menopausal status must be considered. Including menopausal status as a covariate may reduce the 'noise' effects upon maternal BMD from rapid bone loss in comparisons with BMD of offspring, in whom BMD is mainly determined by peak bone mass.

Osteoporosis remains a major public health problem, with substantial morbidity and mortality. Therapeutic options to improve BMD after a fracture has occured are limited in efficacy. It is my hope that the work presented in this thesis will improve understanding of the pathology of this debilitating illness, and that this may lead to better prevention and treatment of osteoporosis.

Appendix 1: Publications

Papers

E L Duncan, M A Brown, J S Sinsheimer, J I Bell, A J Carr, B P Wordsworth, J A H Wass (1999) "Suggestive Linkage of the Parathyroid Receptor Type 1 to Osteoporosis" J Bone Miner Res 14(12):1993 – 99.

N Carter, E L Duncan and B P Wordsworth (2000) "Bone mineral density in adults with Marfan syndrome" Rheumatology (Oxford) 39(3) 307-9.

Invited Reviews

M A Brown and E L Duncan (1999) "Genetic studies of osteoporosis" Expert Reviews in Molecular Medicine http://www-ermm.cbcu.cam.ac.uk/.

Oral Presentations at Scientific Meetings

E L Duncan, M A Brown, J S Sinsheimer, J I Bell, A J Carr, B P Wordsworth, J A H Wass: "A large candidate gene study in families with osteoporosis – suggestive linkage to the Parathyroid Hormone Receptor type 1" presented at the American Society of Bone and Mineral Research 20th Annual Meeting, San Francisco, USA, 1998.

E L Duncan, M A Brown, J S Sinsheimer, J I Bell, A J Carr, B P Wordsworth, J A H Wass: "Suggestive linkage of PTHR1 to osteoporosis" presented at the American Endocrine Society 81st Annual Meeting, San Diego, USA, 1999.

E L Duncan, M A Brown, J S Sinsheimer, J I Bell, A J Carr, B P Wordsworth, J A H Wass: "And the hip bone"s connected to the....a linkage study in osteoporosis" presented at the Annual Scientific Meeting, Royal Australasian College of Physicians, Perth, Australia, 1999.

E L Duncan, L Cardon, J A H Wass and M A Brown: "Site and Gender Specificity of the Genetic Control of Bone Density" presented at the American Society of Bone and Mineral Research 22nd Annual Meeting, Toronto, Canada, 2000.

Abstracts

E L Duncan, L Cardon, J A H Wass and M A Brown (2000) "Site and Gender Specificity of the Genetic Control of Bone Density" J Bone Miner Res 15 (Suppl 1) S163.

E L Duncan, L Lonie, J A H Wass and M A Brown (2000) "Linkage Mapping and Mutation Screening of the PTHR1 locus in Osteoporosis" J Bone Miner Res 15 (Suppl 1) S491.

E L Duncan, J A H Wass and M A Brown (2000) "Linkage studies implicate OPGL/TRANCE but not OPG or RANK in the control of bone density" J Bone Miner Res 15 (Suppl 1) S214.

E L Duncan, M Olavesen, J A H Wass and M A Brown (2000) "Parental Origin Effect on Association with Polymorphisms of COL1A1" J Endocrinol 164 (Suppl 1).

E L Duncan, J A H Wass, M A Brown (2000) "Linkage mapping and mutation screening of the PTHR1 locus in osteoporosis" Rheumatology 39 (Suppl 1) S175.

E L Duncan, M Olavesen, J A H Wass, M A Brown (2000) "COL1A1 and osteoporosis – within-family association studies suggest parent of origin effect" Rheumatology; 39 (Suppl 1) 102.

E L Duncan, M Olavesen, J A H Wass, M A Brown (2000) "The Sp1 COL1A1 gene polymorphism and bone density – association in the elderly implies role in determining bone loss" Rheumatology 39 (Suppl 1).

E L Duncan, L Lonie, J A H Wass, M A Brown (1999) "Mutations in the parathyroid hormone receptor type 1 – detection using denaturing high performance liquid chromatography in patients with osteoporosis" J Endocrinol 163(Suppl 1): S8.

E L Duncan, M A Brown, M Olavesen, J A H Wass (1999) "Is BMD associated with polymorphisms of COL1A1?" J Endocrinol 163 (Suppl 1): S9.

E L Duncan, M A Brown, J S Sinsheimer, A J Carr, B P Wordsworth, J A H Wass (1999) "Linkage of PTHR1 with Osteoporosis", Endocrinology 140 (Suppl 1) OR23-4.

E L Duncan, M A Brown, J S Sinsheimer, A J Carr, B P Wordsworth, J A H Wass (1999) "A Candidate Gene Linkage Screen in Osteoporosis" Aust NZ J Med Vol 30.

E L Duncan, M A Brown, J Sinsheimer, J Bell, A J Carr, B P Wordsworth, J A H Wass (1998) "Suggestive linkage of the parathyroid receptor type 1 to osteoporosis" Bone 23(5) Suppl S160.

N Carter, E Duncan and P Wordsworth (1998) "Osteoporosis in Marfan"s syndrome" Arthritis and Rheumatism 41(9) Suppl S305.

J S Sinsheimer, D E Weeks, E L Duncan, G M Lathrop (1997) "Designing a linkage study using bone mineral density as a surrogate marker for osteoporosis." Am J Hum Gen, 61(4) Suppl A294.

E L Duncan, J Sinsheimer, K Dymott, B Jakobsen, A J Shipman, J A H Wass (1997) "Bone mineral density of relatives of male and female patients with primary osteoporosis.": J Endocrinol, 152 (Suppl 1) S11.

E L Duncan, J Sinsheimer, K Dymott, B Jakobsen, A J Shipman and J A H Wass (1997) "Low bone mineral density of relatives of male and female patients with primary osteoporosis" Endocrinology 138 (Suppl 1) S496.

Appendix 4.3:

Two point and Multipoint Results for Microsatellite Markers from Chapter 4 Candidate Gene Linkage Study

			Maximum LOD score LS		Maximum LOD score FN	
Candidate	**Marker**	**cM from p telomere**	**Two-point**	**Multipoint**	**Two-point**	**Multipoint**
AR	dxs1275	93.5	0.0	0.0	0.0	0.7
AR	dxs986	95.9	0.0	0.0	0.5	0.6
CSR	d3s1309	157	0.0	0.0	0.0	0.0
CSR	d3s1593	165	0.0	0.0	0.6	0.1
CSR	d3s1279	173	0.2	0.0	0.1	0.1
CSR	d3s1268	180	0.0	0.0	0.0	0.0
COL1A1	d17s791	65	0.0	0.0	0.3	0.2
COL1A1	d17s1604	82.9	0.0	0.0	0.6	0.5
COL1A1	d17s807	87.6	0.5	0.3	1.7	0.6
COL1A1	d17s789	90.8	0.0	0.3	0.1	0.5
COL1A2/CR	d12s1702	78.9	0.1	0.4	0.9	0.6
COL1A2/CR	d7s2431	109	0.1	0.6	0.4	0.3
COL1A2/CR	col1a2	intragenic	0.6	0.7	0.2	0.3
COL2A1/VDR	d12s368	67.3	0.5	0.6	0.2	0.4
COL2A1/VDR	d12s1586	70.6	0.7	0.7	1.0	0.4
COL2A1/VDR	d12s83	76.5	1.7	1.1	0.0	0.5
CSF-1	d1s209	95.9	0.0	0.0	0.2	0.1
CSF-1	d1s198	103	0.0	0.0	0.5	0.1
CSF-1	d1s216	107	0.1	0.0	0.0	0.1
CSF-1	d1s207	118	0.1	0.1	0.2	0.7

			Maximum LOD score LS		Maximum LOD score FN	
Candidate	**Marker**	**cM from p telomere**	**Two-point**	**Multipoint**	**Two-point**	**Multipoint**
EGF	d4s1572	106	0.0	0.1	0.0	0.1
EGF	d4s406	116	0.0	0.0	0.0	0.3
EGF	d4s193	117	0.0	0.0	0.2	0.5
EGF	d4s430	125	0.4	0.3	1.3	1.4
EGF	d4s429	131	0.2	0.3	1.8	1.6
EGF	d4s247	*	0.0	0.3	0.0	1.4
ER-α	d6s1654	151	0.1	0.1	0.0	0.1
ER-α	d6s441	155	0.2	0.8	0.0	0.4
ER-α	d6s1577	158	0.5	1.4	0.1	0.4
ER-α	ER	intragenic				
fibrillin	mts1	intragenic	0.1	0.0	1.0	0.3
fibrillin	mts4	intragenic	0.0	0.0	0.2	0.3
IGF-1	d12s78	113	0.0	0.0	0.5	0.6
IGF-1	d12s79	126	0.0	0.0	0.1	1.2
IGF-1	d12s86	135	0.0	0.0	0.0	1.4
IGF-1	IGF-1	intragenic	0.6	0.6	0.8	0.8
IL-1	d2s160	127	0.7	0.4	0.5	1.4
IL-1	d2s2265	134	0.7	0.0	0.3	0.1
IL-1	IL-1	intragenic	0.3	0.0	0.3	0.0
IL-11	d19s412	69.9	0.5	0.3	0.1	0.0
IL-11	d19s866	81.1	0.1	0.3	0.0	0.0
IL-4	d5s2057	135	0.0	0.0	1.1	0.3
IL-4	d5s393	141	0.0	0.1	0.1	0.8
IL-4	d5s2017	145	0.3	0.2	1.2	0.9
IL-4	d5s178	*	0.0	0.2	0.1	1.1

			Maximum LOD score LS		Maximum LOD score FN	
Candidate	**Marker**	**cM from p telomere**	**Two-point**	**Multipoint**	**Two-point**	**Multipoint**
IL-6	d7s503	29.6	1.2	1.1	0.6	0.3
IL-6	d7s493	35	0.3	0.9	0.2	0.5
IL-6	d7s673	38.8	0.3	0.7	0.4	0.6
osteocalcin	d1s2815	194	0.0	0.0	0.0	0.0
osteocalcin	d1s238	207	0.0	0.0	0.0	0.0
osteopontin	d4s392	77.9	0.0	0.1	0.0	0.0
osteopontin	d4s3042	81.9	0.0	0.0	0.0	0.0
osteopontin	d4s395	90.8	0.3	0.6	0.2	0.5
PTH	d11s902	24.7	0.1	0.3	0.4	0.5
PTH	d11s1755	30.3	0.7	0.6	0.6	0.4
PTH	d11s915	34.3	0.5	0.3	0.0	0.1
PTHR1	d3s3559	62.7	1.3	0.8	1.5	1.8
PTHR1	d3s1289	69.1	0.3	0.4	2.7	2.0
PTHrP	d12s364	31.7	0.0	0.0	0.0	0.0
PTHrP	d12s1699	37.3	0.0	0.0	0.1	0.0
TGF-β	d19s422	62.5	0.0	0.1	0.0	0.0
TNF	d6s276	44.9	0.2	0.2	0.3	0.2
TNF	d6s1583	47.6	0.7	0.2	0.8	0.3
TNF	TNF	intragenic	0.1	0.3	0.0	0.2

*no formal definitive location in Kosambi cM. However both markers (d5s178 and d4s178) were mapped using MapMaker/Sibs to enable multipoint analysis.

Abbreviations used above not used elsewhere:

CSR Calcium sensing receptor

CR Calcitonin receptor

AR Androgen receptor

Appendix 5.4.2:

Primers and Optimum Reaction Conditions for Exons and Promoter regions of PTHR1

All reactions were carried out using oil-free PCR with sealed lids on the reaction plates.

Exon	Size (bp)	[Mg2+] (mmol/L)	T_m	Addition	Forward primer	Reverse primer
S	199	1.5	58	Deaza-dGTP	gccagcctgacgcagctctgca	actgcgtgccttagacctactc
E1	180	1.5	58		ccggaaagtcctgcctgtggtc	tgacctcatacccagaccctct
E2	220	2.5	58		acagctgacagccatcattacc	gtggatccaagcccatgccagc
E3	180	2	55		ggtatcccctaccctgtctgtc	ctccttgtaatccccacccctt
G	201	2.5	55		tcgagacctccctgccggccc	gaatttatctggtcaggttgg
M1	205	0.5	61		agggctctgactgtgtctcc	accatgtcccgccgcctctc
M2	276	0.5	61		cttcctgtccacccaccgc	gcagaggggtactcacgta
M3	267	2	58		acttcccggaggcaggccctgc	tctccctgtcacccacgggtcc
M4	191	1.5	58		gaatgaccttgtggacagcagc	ctcacatgcttcctggaagaaa
El2	204	1.5	58		cctgtgtcctcaacagcta	cttggtggccagcagccca
M5	236	1.5	58		actagggtgcagcctccagacg	aggatcattcatgggccactg
M6-7	340	1.5	55		ggaagtggcgttggccctgacc	ccctgccctatgccaacactgt
T1	340	2.5	58		agacacacctgactgccgcacc	agccgttgaggaacccatcgtc
T2	260	3	58		gccctggagaccctcgagacca	tgtttttcctcttggccccagg
U1	324	3	55		tgggcatctgaaacaccggca	agctgtgctcaggcccctag
U2	343	1.5	58		cttgggcttgacagatttgc	atgcctggagcgcagggcttta
U3	362	0.5	59	DMSO	cctctcggcctctccacact	gactccggccacttcccctc
New M6-7	256	1.5	55		ggcaagtccagatgcactatg	cagggtggaagaatggag

Appendix 5.4.3: Promoter Region U3 Sequence: Comparisons

1. Comparison of U3 Sequence Obtained by E.Duncan and Sequence Released by the Human Genome Project

Sequence 1 from Human Genome Project (http://genome.ucsc.edu).

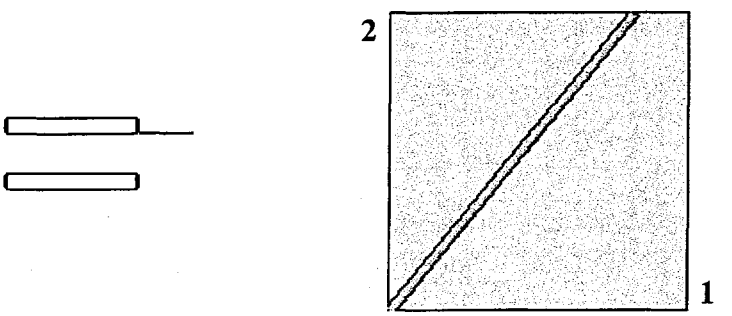
Sequence 2 from E.Duncan.

Comparison of sequences made using 'Blast2 programme

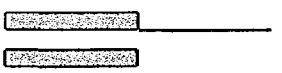
(http://www.ncbi.nih.gov/blast.bl2seq/wblast2.cgi).

Sequence 1 HGP **Length** 451 (1 .. 451)

Sequence 2 ED **Length** 369 (1 .. 369)



Score = 426 bits (268), Expect = e-117
Identities = 368/369 (99%)
Strand = Plus / Plus



Query: 1 cctctcggcctctccacactcccgcgtcggcggctnnnnnnnnnnnnnnnnnnnnnnnn 60

Sbjct: 1 cctctcggcctctccacactcccgcgtcggcggctgcggaggggtgggggcgggagagg 60

Query: 61 nnnnnnnnnnnnnnnnnnnnnaagaggcgcccggccggggagaaggggagcggcagacg 120

Sbjct: 61 cccgggagggcgcgggggagggaagaggygcccggccggggagaaggggagcggcagacg 120

Query: 121 ccgaggcgaggganmnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnc 180

|

Sbjct: 121 ccgaggcgagggatgcgcgcggcgggcggtggctccgagcggcggccgggcggggggcgc 180

Query: 181 tggaggccaggccggccagcggggggtatcccgagagctccatgaagtccccccggggcc 240

Sbjct: 181 tggaggccaggccggccagcggggggtatcccgagagctccatgaagtccccccggggcc 240

Query: 241 gcggacggggcgctggcttggggaggctgtcnnnnnnnccccgacatccatggcaaggcg 300

Sbjct: 241 gcggacggggcgctggcttggggaggctgtcggggggggccccgacatccatggcaaggcg 300

Query: 301 ggggccgcggcggcgcgctcggagtaagtcggggctggggacccgcgccgaggggaagtg 360

Sbjct: 301 ggggccgcggcggcgcgctcggagtaagtcggggctggggacccgcgccgaggggaagtg 360

Query: 361 gccggagtc 369

Sbjct: 361 gccggagtc 369

2. Comparison of previously published U3 Sequence and Sequence Released by the Human Genome Project

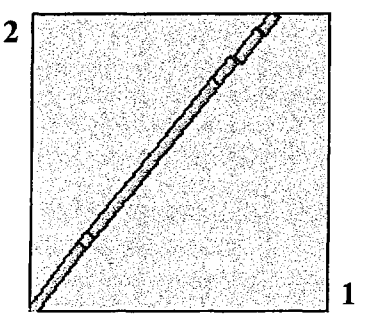
Sequence 1 from Human Genome Project (http://genome.ucsc.edu).

Sequence 2 from previously published data (Bettoun et al. 1998).

Comparison of sequences made using 'Blast2 programme (http://www.ncbi.nih.gov/blast.bl2seq/wblast2.cgi).

Sequence 1 HGP **Length** 451 (1 .. 451)

Sequence 2 Bettoun **Length** 362 (1 .. 362)



Score = 329 bits (207), Expect = 6e-88
Identities = 354/371 (95%), Gaps = 11/371 (2%)
Strand = Plus / Plus

Query: 1 cctctcggcctctccacactcccgcgtcggcggctnnnnnnnnnnnnnnnnnnnnnnnn 60

Sbjct: 1 cctctcggcctctccacactcccgcgtcggcggctgcggaggggtgggggcgggagagg 60

Query: 61 nnnnnnnnnnnnnnnnnnnnn-aagaggcgcc-cggccggggagaaggggagcggcaga 118

Sbjct: 61 cccgggagggcgccgggggagggaagaggcgcccggcccggggagaaggggagcggcaga 120

Query: 119 cgccgaggcgagggannnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn 178

Sbjct: 121 cgccgaggcgagggatgcgcgcggcgggcggtggctccgagcggcggccgggcgggggggc 180

Query: 179 nctggaggccaggccggccagcgggggtatcccgagagctccatgaagtccccccgggg 238

Sbjct: 181 gctggaggccaggccggccagcgggggtatcccgagagctccatgaagtccccccgggg 240

Query: 239 ccgcggacggggcgctggcttggggaggctgtcnnnnnnnccccgacatccatggcaagg 298

Sbjct: 241 ccgcggacggggcgctggcttggggaggctgtcgggggggg-cccgacatccatggcaagg 299

Query: 299 cgggggccgcggcggcgcgctcggagtaagtcggggctggggacccgcgccgaggggaag 358

Sbjct: 300 cgggggc-------gggcgctcggagtaagtcggggctggggacccgc-ccgaggggaag 351

Query: 359 tggccggagtc 369

Appendix 5.4.4:

Comparison of Previously Published U4 Sequence with Sequence Released by the Human Genome Project

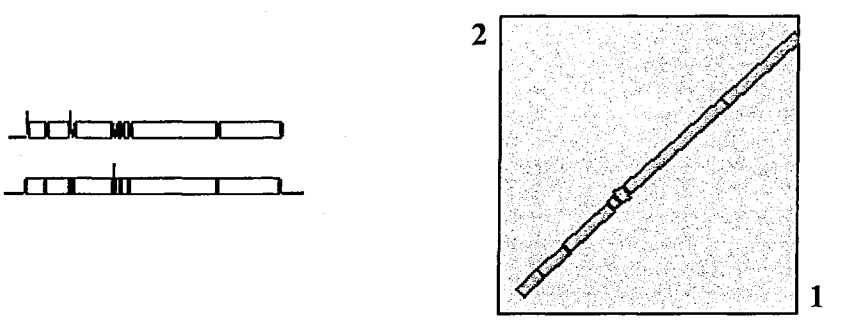
Sequence 1 from previously published data (Bettoun et al. 1998).

Sequence 2 from the Human Genome Project (http://genome.ucsc.edu).

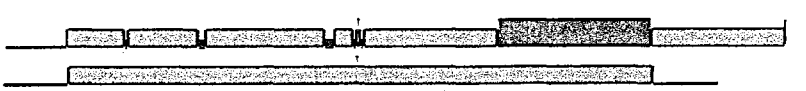
Comparison of sequences made using 'Blast2' programme (http://www.ncbi.nih.gov/blast.bl2seq/wblast2.cgi).

Sequence 1 Bettoun **Length** 618 (1 .. 618)

Sequence 2 HGP **Length** 690 (1 .. 690)



Score = 611 bits (385), Expect = e-172
Identities = 545/590 (92%), Gaps = 19/590 (3%)
Strand = Plus / Plus



Query: 48 gcggcgcgggannnnnnnnnnnnnnnnnnnnnnnnnnnnnnnncc-cggcatatggatgtg 106

Sbjct: 52 gcggcgcgggaggggggcgggggcgggccgggggaggcgggcccggccatatggatgtg 111

Query: 107 atttcttcgctccgaggcagacgggcccgtccgcagcg------gcttggcgcccgccgn 160

Sbjct: 112 atttcttcgctccgaggcagacgggccgctccgcagcgctcggcgcccgcccgccgcccg 171

Query: 161 nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnttgctcgt 220

Sbjct: 172 cccggcctccggctctccctccctccctcctgtccctccctccctccctcctttgcgctg 231

Query: 221 cgtcgtcgctcgct--------cgccctcagcgcat-ggccccgcgccgggcccc-gggc 270

Sbjct: 232 ctcgctcgctcgctcgctcgctcgccctcagcgcatgggccccgcgccgggccccggggc 291

Query: 271 ctcgggccgccgggacgccggggtcccataggccggggcgtgggcggggcggccagcctg 330

Sbjct: 292 ctcgggccgccgggacgccggggtcccataggccggggcgtgggcggggcggccagcctg 351

Query: 331 acgcagctctgcacccccctaccaccaccagggccggcggcggcggctgccccgagggacg 390

Sbjct: 352 acgcagctctgcacccccctaccaccaccagggccggcggcggcggctgccccgagggacg 411

Query: 391 cggccctaggcggtggcgatggggaccgcccggatcgcacccggcctggcgctcctgctc 450

Sbjct: 412 cggccctaggcggtggcgatggggaccgcccggatcgcacccggcctggcgctcctgctc 471

Query: 451 tgctgccccgtgctcagctc--cgtacgcgctggtgagtcccccgccgccaacactccgg 508

Sbjct: 472 tgctgccccgtgctcagctccgcgtacgcgctggtgagtcccccgccgccaacactccgg 531

Query: 509 gacaggctgcgggcttaccctagggtccgcgggataggtctaaggcacgcagtcttgagt 568

Sbjct: 532 gacaggctgcgggcttaccctagggtccgcgggataggtctaaggcacgcagtcttgagt 591

Query: 569 tcccccagtagttcgaactttgggtgagagtcccctctgatccaggatcc 618

Sbjct: 592 tcccccagtagttcgaactttgggtgagagtcccctctgatccaggatcc 641

References

Abecasis, G. R., L. R. Cardon and W. O. Cookson (2000). "A general test of association for quantitative traits in nuclear families." Am J Hum Genet 66(1): 279-92.

Abecasis, G. R., E. Noguchi, A. Heinzmann, J. A. Traherne, S. Bhattacharyya, N. I. Leaves, G. G. Anderson, Y. Zhang, N. J. Lench, A. Carey, L. R. Cardon, M. F. Moffatt and W. O. Cookson (2001). "Extent and Distribution of Linkage Disequilibrium in Three Genomic Regions." Am J Hum Genet 68(1): 191-197.

Abou-Samra, A. B., H. Jüppner, T. Force, M. W. Freeman, X. F. Kong, E. Schipani, P. Urena, J. Richards, J. V. Bonventre, J. T. Potts, Jr. and et al. (1992). "Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium." Proc Natl Acad Sci U S A 89(7): 2732-6.

al-Aqeel, A., P. Ozand, S. Sobki, W. Sewairi and S. Marx (1993). "The combined use of intravenous and oral calcium for the treatment of vitamin D dependent rickets type II (VDDRII)." Clin Endocrinol (Oxf) 39(2): 229-37.

Albagha, O. M., F. E. McGuigan, D. M. Reid and S. H. Ralston (2001). "Estrogen receptor alpha gene polymorphisms and bone mineral density: haplotype analysis in women from the United Kingdom." J Bone Miner Res 16(1): 128-34.

Allison, D. B. (1997). "Transmission-disequilibrium tests for quantitative traits." Am J Hum Genet 60(3): 676-90.

Allison, D. B. (1997). "Transmission-disequilibrium tests for quantitative traits [published erratum appears in Am J Hum Genet 1997 Jun;60(6):1571]." Am J Hum Genet 60(3): 676-90.

Allison, D. B., M. Heo, N. Kaplan and E. R. Martin (1999). "Sibling-based tests of linkage and association for quantitative traits." Am J Hum Genet 64(6): 1754-63.

Allison, D. B., M. Heo, N. J. Schork, S. L. Wong and R. C. Elston (1998). "Extreme selection strategies in gene mapping studies of oligogenic quantitative traits do not always increase power." Hum Hered 48(2): 97-107.

Almasy, L. and J. Blangero (1998). "Multipoint quantitative-trait linkage analysis in general pedigrees." .

Aloia, J. F., D. McGowan, E. Erens and G. Miele (1992). "Hip fracture patients have generalized osteopenia with a preferential deficit in the femur." Osteoporos Int 2(2): 88-93.

Ames, S., K. Ellis, S. Gunn, K. Copeland and S. Abrams (1999). "Vitamin D receptor gene FokI polymorphism predicts calcium absorptio and bone mineral density in children." J Bone Miner Res 14(5): 740-746.

Amizuka, N., J. E. Henderson, J. H. White, A. C. Karaplis, D. Goltzman, T. Sasaki and H. Ozawa (2000). "Recent studies on the biological action of parathyroid hormone (PTH)-related peptide (PTHrP) and PTH/PTHrP receptor in skeletal tissue." Histol Histopathol 15(3): 957-70.

Amizuka, N., M. Y. Kwan, D. Goltzman, H. Ozawa and J. H. White (1999). "Vitamin D3 differentially regulates parathyroid hormone/parathyroid hormone-related peptide receptor expression in bone and cartilage." J Clin Invest 103(3): 373-81.

Amizuka, N., H. S. Lee, M. Y. Kwan, A. Arazani, H. Warshawsky, G. N. Hendy, H. Ozawa, J. H. White and D. Goltzman (1997). "Cell-specific expression of the parathyroid hormone (PTH)/PTH-related peptide receptor gene in kidney from kidney-specific and ubiquitous promoters." Endocrinology 138(1): 469-81.

Ammann, P., R. Rizzoli, J. P. Bonjour, S. Bourrin, J. M. Meyer, P. Vassalli and I. Garcia (1997). "Transgenic mice expressing soluble tumor necrosis factor-receptor are protected against bone loss caused by estrogen deficiency." J Clin Invest 99(7): 1699-703.

Amos, C., D. Zhu and E. Boerwinkle (1996). "Assessing genetic linkage and association with robust components of variance approaches." Am J Hum Genet 60: 143-160.

Amos, C. I., J. Krushkal, T. J. Thiel, A. Young, D. K. Zhu, E. Boerwinkle and M. de Andrade (1997). "Comparison of model-free linkage mapping strategies for the study of a complex trait." Genet Epidemiol 14(6): 743-8.

Anderson, D. M., E. Maraskovsky, W. L. Billingsley, W. C. Dougall, M. E. Tometsko, E. R. Roux, M. C. Teepe, R. F. DuBose, D. Cosman and L. Galibert (1997). "A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function." Nature 390(6656): 175-9.

Arai, H., K. Miyamoto, Y. Taketani, H. Yamamoto, Y. Iemori, K. Morita, T. Tonai, T. Nishisho, S. Mori and E. Takeda (1997). "A vitamin D receptor gene polymorphism in the translation initiation codon: effect on protein activity and relation to bone mineral density in Japanese women." J Bone Miner Res 12(6): 915-21.

Arden, N. K., J. Baker, C. Hogg, K. Baan and T. D. Spector (1996). "The heritability of bone mineral density, ultrasound of the calcaneus and hip axis length: a study of postmenopausal twins." J Bone Miner Res 11(4): 530-4.

Arnold, A., S. A. Horst, T. J. Gardella, H. Baba, M. A. Levine and H. M. Kronenberg (1990). "Mutation of the signal peptide-encoding region of the preproparathyroid hormone gene in familial isolated hypoparathyroidism." J Clin Invest 86(4): 1084-7.

Asou, Y., S. Rittling, H. Yoshitake, K. Tsuji, K. Shinomiya, A. Nifuji, D. Denhardt and M. Noda (2001). "Osteopontin facilitates angiogenesis, accumulation of osteoclasts, and resorption in ectopic bone." Endocrinology 142(3): 1325-32.

Baccarini-Contri, M., F. Taparelli and I. Pasquali-Ronchetti (1994). "Osteopontin is a constitutive component of normal elastic fibers in human skin and aorta." Matrix Biol. 14: 553-60.

Badner, J., E. Gershon and L. Goldin (1998). "Optimal Ascertainment Strategies to Detect Linkage to Common Disease Alleles." Am J Hum Genet 63: 880-888.

Bagger, Y. Z., H. L. Jorgensen, A. M. Heegaard, L. Bayer, L. Hansen and C. Hassager (2000). "No major effect of estrogen receptor gene polymorphisms on bone mineral density or bone loss in postmenopausal Danish women." Bone 26(2): 111-6.

Bailly, S., N. Israel, M. Fay, M. A. Gougerot-Pocidalo and G. W. Duff (1996). "An intronic polymorphic repeat sequence modulates interleukin-1 alpha gene regulation." Mol Immunol 33(11-12): 999-1006.

Balsan, S., M. Garabedian, M. Larchet, A. M. Gorski, G. Cournot, C. Tau, A. Bourdeau, C. Silve and C. Ricour (1986). "Long-term nocturnal calcium infusions can cure rickets and promote normal mineralization in hereditary resistance to 1,25-dihydroxyvitamin D." J Clin Invest 77(5): 1661-7.

Barger Lux, M. J., R. P. Heaney, J. Hayes, H. F. DeLuca, M. L. Johnson and G. Gong (1995). "Vitamin D receptor gene polymorphism, bone mass, body size, and vitamin D receptor density." Calcif Tissue Int 57(2): 161-2.

Barker, D. J. P. (1995). "Fetal origins of coronary heart disease." BMJ 311:171-4.

Barrett-Connor, E. and D. Goodman-Gruen (1998). "Gender differences in insulin-like growth factor and bone mineral density association in old age: the Rancho Bernardo Study." J Bone Miner Res 13(8): 1343-9.

Bass, S., P. D. Delmas, G. Pearce, E. Hendrich, A. Tabensky and E. Seeman (1999). "The differing tempo of growth in bone size, mass, and density in girls is region-specific." J Clin Invest 104(6): 795-804.

Bauer, D. C., C. C. Gluer, J. A. Cauley, T. M. Vogt, K. E. Ensrud, H. K. Genant and D. M. Black (1997). "Broadband ultrasound attenuation predicts fractures strongly and independently of densitometry in older women. A prospective study. Study of Osteoporotic Fractures Research Group." Arch Intern Med 157(6): 629-34.

Bauer, D. C., C. C. Gluer, H. K. Genant and K. Stone (1995). "Quantitative ultrasound and vertebral fracture in postmenopausal women. Fracture Intervention Trial Research Group." J Bone Miner Res 10(3): 353-8.

Becherini, L., L. Gennari, L. Masi, R. Mansani, F. Massart, A. Morelli, A. Falchetti, S. Gonnelli, G. Fiorelli, A. Tanini and M. L. Brandi (2000). "Evidence of a linkage disequilibrium between polymorphisms in the human estrogen receptor alpha gene and their relationship to bone mass variation in postmenopausal Italian women." Hum Mol Genet 9(13): 2043-50.

Bell, G. I., S. Horita and J. H. Karam (1984). "A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus." Diabetes 33(2): 176-83.

Bellido, T., R. L. Jilka, B. F. Boyce, G. Girasole, H. Broxmeyer, S. A. Dalrymple, R. Murray and S. C. Manolagas (1995). "Regulation of interleukin-6, osteoclastogenesis, and bone mass by androgens. The role of the androgen receptor." J Clin Invest 95(6): 2886-95.

Berg, J. P., J. A. Falch and E. Haug (1996). "Fracture rate, pre- and postmenopausal bone mass and early and late postmenopausal bone loss are not associated with vitamin D receptor genotype in a high-endemic area of osteoporosis." Eur J Endocrinol 135(1): 96-100.

Berg, J. P., E. H. Lehmann, J. A. Stakkestad, E. Haug and J. Halse (2000). "The Sp1 binding site polymorphism in the collagen type I alpha 1 (COLIA1) gene is not associated with bone mineral density in healthy children, adolescents, and young adults." Eur J Endocrinol 143(2): 261-5.

Bertoldo, F., L. D'Agruma, F. Furlan, F. Colapietro, M. T. Lorenzi, N. Maiorano, A. Iolascon, L. Zelante, V. Locascio and P. Gasparini (2000). "Transforming growth factor-beta1 gene polymorphism, bone turnover, and bone mass in Italian postmenopausal women." J Bone Miner Res 15(4): 634-9.

Bertolini, D. R., G. E. Nedwin, T. S. Bringman, D. D. Smith and G. R. Mundy (1986). "Stimulation of bone resorption and inhibition of bone formation in vitro by human tumour necrosis factors." Nature 319(6053): 516-8.

Bettoun, J. D., M. Y. Kwan, M. Minagawa, L. C. Alpert, C. G. Goodyer, G. N. Hendy, D. Goltzman and J. H. White (2000). "Methylation patterns of human parathyroid hormone (PTH)/PTH-related peptide receptor gene promoters are established several weeks prior to onset of their function." Biochem Biophys Res Commun 267(2): 482-7.

Bettoun, J. D., M. Minagawa, G. N. Hendy, L. C. Alpert, C. G. Goodyer, D. Goltzman and J. H. White (1998). "Developmental upregulation of human parathyroid hormone (PTH)/PTH-related peptide receptor gene expression from conserved and human-specific promoters." J Clin Invest 102(5): 958-67.

Bettoun, J. D., M. Minagawa, M. Y. Kwan, H. S. Lee, T. Yasuda, G. N. Hendy, D. Goltzman and J. H. White (1997). "Cloning and characterization of the promoter regions of the human parathyroid hormone (PTH)/PTH-related peptide receptor gene: analysis of deoxyribonucleic acid from normal subjects and patients with pseudohypoparathyroidism type 1b." J Clin Endocrinol Metab 82(4): 1031-40.

Bilezikian, U. and S. Silverberg (2000). Parathyroid hormone: Does It Have a Role in the Pathogenesis of Osteoporosis? Osteoporosis and Metabolic Bone Disease. C. Rosen. Philadelphia, W.B. Saunders Company. 20: 559-568.

Bilous, R. W., G. Murty, D. B. Parkinson, R. V. Thakker, M. G. Coulthard, J. Burn, D. Mathias and P. Kendall-Taylor (1992). "Brief report: autosomal dominant familial hypoparathyroidism, sensorineural deafness, and renal dysplasia." N Engl J Med 327(15): 1069-74.

Bizzarri, C., A. Shioi, S. L. Teitelbaum, J. Ohara, V. A. Harwalkar, J. M. Erdmann, D. L. Lacey and R. Civitelli (1994). "Interleukin-4 inhibits bone resorption and acutely increases cytosolic Ca2+ in murine osteoclasts." J Biol Chem 269(19): 13817-24.

Black, D. and C. Cooper (2000). Epidemiology of fractures and assessment of fracture risk. Osteoporosis and metabolic bone disease. C. J. Rosen. Philadelphia, W B Saunders Company. 20:3: 439-453.

Blomstrand, S., I. Claesson and J. Save-Soderbergh (1985). "a case of lethal congential dwarfism with accelerated skeletal maturation." Pediatr Radiol 15: 141-143.

Boonen, S., J. Aerssens, J. Dequeker, P. Nicholson, X. Cheng, G. Lowet, G. Verbeke and R. Bouillon (1997). "Age-associated decline in human femoral neck cortical and trabecular content of insulin-like growth factor I: potential implications for age-related (type II) osteoporotic fracture occurrence." Calcif Tissue Int 61(3): 173-8.

Brown, M. A., M. A. Haughton, S. F. A. Grant, A. S. Gunnell, N. K. Henderson and J. A. Eisman (2001). "The Genetic Control of Bone Density and Turnover – The Role of the COL1A1, Estrogen Receptor and VDR Genes." J Bone Miner Res 16(4): (in press).

Brown, M. A., L. G. Kennedy, A. J. MacGregor, C. Darke, E. Duncan, J. L. Shatford, A. Taylor, A. Calin and P. Wordsworth (1997). "Susceptibility to ankylosing spondylitis in twins: the role of genes, HLA, and the environment." Arthritis Rheum 40(10): 1823-8.

Brown, M. A., S. H. Laval, S. Brophy and A. Calin (2000). "Recurrence risk modelling of the genetic susceptibility to ankylosing spondylitis." Ann Rheum Dis 59(11): 883-6.

Bucay, N., I. Sarosi, C. R. Dunstan, S. Morony, J. Tarpley, C. Capparelli, S. Scully, H. L. Tan, W. Xu, D. L. Lacey, W. J. Boyle and W. S. Simonet (1998). "osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification." Genes Dev 12(9): 1260-8.

Burger, H., P. van Daele, D. algra, F. van den Ouweland, D. Grobbee, A. Hofman, C. van Kuijk, H. Schuette, J. Birkenhaeger and H. Pols (1994). "The assocaiton between age and bone mineral density in men adn women aged 55 years and over: the Rotterdam Study." Bone and Mineral 25: 1-13.

Burgess, T. L., Y. Qian, S. Kaufman, B. D. Ring, G. Van, C. Capparelli, M. Kelley, H. Hsu, W. J. Boyle, C. R. Dunstan, S. Hu and D. L. Lacey (1999). "The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts." J Cell Biol 145(3): 527-38.

Burich, H., L. Wolf and R. e. a. Budde (1992). "Androstenidione metabolism in cultured human osteoblast like cells." J Clin Endocrinol Metab 75: 101-105.

Camp, N. J. (1997). "Genomewide transmission/disequilibrium testing--consideration of the genotypic relative risks at disease loci." Am J Hum Genet 61(6): 1424-30.

Carani, C., K. Qin, M. Simoni, M. Faustini-Fustini, S. Serpente, J. Boyd, K. S. Korach and E. R. Simpson (1997). "Effect of testosterone and estradiol in a man with aromatase deficiency." N Engl J Med 337(2): 91-5.

Cardon, L., D. Fulker and S. Cherny (1995). "Linkage Analysis of a Common Oligogenic disease Using Selected Sib Pairs." Genetic Epidemiology 12: 741-746.

Cardon, L. R. and D. W. Fulker (1994). "The power of interval mapping of quantitative trait loci, using selected sib pairs." Am J Hum Genet 55(4): 825-33.

Carey, G. and J. Williamson (1991). "Linkage analysis of quantitative traits: increased power by using selected samples." Am J Hum Genet 49(4): 786-96.

Cargill, M., D. Altshuler, J. Ireland, P. Sklar, K. Ardlie, N. Patil, N. Shaw, C. R. Lane, E. P. Lim, N. Kalyanaraman, J. Nemesh, L. Ziaugra, L. Friedland, A. Rolfe, J. Warrington, R. Lipshutz, G. Q. Daley and E. S. Lander (1999). "Characterization of single-nucleotide polymorphisms in coding regions of human genes." Nat Genet 22(3): 231-8.

Carter, D. R., M. L Bouxsein and R. Marcus (1992) "New approaches for interpreting projected bone densitometry data." J Bone Miner Res 7(2): 137-145.

Carter, N., E. Duncan and P. Wordsworth (2000). "Bone mineral density in adults with Marfan syndrome." Rheumatology (Oxford) 39(3): 307-9.

Cauley, J., J. Zmuda, L. Palermo, K. Stone, D. Black and M. Nevitt (2000). "Do men and women fracture at the same BMD level?" J Bone Miner Res 2000(Suppl 1): S144.

Center, J. R., T. V. Nguyen, D. Schneider, P. N. Sambrook and J. A. Eisman (1999). "Mortality after all major types of osteoporotic fracture in men and women: an observational study." Lancet 353(9156): 878-82.

Centrella, M., M. C. Horowitz, J. M. Wozney and T. L. McCarthy (1994). "Transforming growth factor-beta gene family members and bone." Endocr Rev 15(1): 27-39.

Cepollaro, C., S. Gonnelli, C. Pondrelli, S. Martini, A. Montagnani, S. Rossi, L. Gennari and C. Gennari (1997). "The combined use of ultrasound and densitometry in the prediction of vertebral fracture." Br J Radiol 70(835): 691-6.

Chen, J., N. Maruo, T. Kato, K. Hasumi, E. Ogata, M. Shiraki and I. Morita (1995). "Serum levels of soluble interleukin-6 receptor, not interleukin 6 is correlated wtih bone reorption markers and lumbar bone mineral density after menopause." J Bone Miner Res 10(Suppl 1): M378.

Chen, Z., M. Maricic, P. Lund, J. Tesser and O. Gluck (1998). "How the new Hologic hip normal reference values affect the densitometric diagnosis of osteoporosis." Osteoporos Int 8(5): 423-7.

Cheng, W. and K. Tsai (1999). "The Vitamin D receptor start codon polymorphism (Fok1) and bone mineral density in premenopausal women in Taiwan." Osteoporosis Int 9: 545-549.

Cheng, X., G. Lowet, S. Boonen, P. Nicholson, v. d. Perre and J. Dequeqer (1998). "Prediction of vertebral and femoral strength in vitro by bone mineral density measured at different skeletal sites." J Bone Miner Res 13(9): 1439-1443.

Chevalley, T., R. Rizzoli, V. Nydegger, D. Slosman, L. Tkatch, C. H. Rapin, H. Vasey and J. P. Bonjour (1991). "Preferential low bone mineral density of the femoral neck in patients with a recent fracture of the proximal femur." Osteoporos Int 1(3): 147-54.

Chien, H., W. Lin and M. Cho (2000). "Down-regulation of osteoblastic cell differentiation by epidermal growth factor receptor." Calcif Tissue Int 67(2): 141-50.

Chitnavis, J., J. S. Sinsheimer, K. Clipsham, J. Loughlin, B. Sykes, P. D. Burge and A. J. Carr (1997). "Genetic influences in end-stage osteoarthritis. Sibling risks of hip and knee replacement for idiopathic osteoarthritis." J Bone Joint Surg Br 79(4): 660-4.

Choi, Y. M., J. K. Jun, J. Choe, D. Hwang, S. H. Park, S. Y. Ku, D. Kang, J. G. Kim, S. Y. Moon and J. Y. Lee (2000). "Association of the vitamin D receptor start codon polymorphism (FokI) with bone mineral density in postmenopausal Korean women " J Hum Genet 45(5): 280-3.

Christian, J., M. Feinleib, S. Hulley, W. Castelli, R. Fabsitz, R. Garrison, N. Borhani, B. Rosenman and J. Wagner (1974). "Choice of an estimate of genetic variance from twin data." American Journal of Human Genetics 26: 154-161.

Christian, J. C., P.-L. Yu, C. W. Slemenda and C. Conrad Johnston (1989). "Heritability of bone mass: a longitudinal study in aging male twins." Am J Hum Genet 44: 429-433.

Clark, A. G., K. M. Weiss, D. A. Nickerson, S. L. Taylor, A. Buchanan, J. Stengard, V. Salomaa, E. Vartiainen, M. Perola, E. Boerwinkle and C. F. Sing (1998). "Haplotype structure and population genetic inferences from nucleotide-sequence variation in human lipoprotein lipase." Am J Hum Genet 63(2): 595-612.

Clerget-Darpoux, F., C. Bonaiti-Pellie and J. Hochez (1986). "Effects of misspecifying genetic parameters in lod score analysis." Biometrics 42: 393-399.

Cohen-Salal, M. E., C. Baudoin, M. Omouri, D. Kuntz and M. C. De Vernejoul (1998). "Bone mass in middle-aged osteoporotic men and their relatives: familial effect." J Bone Miner Res 13: 1909-1914.

Colvard, D. S., E. F. Eriksen, P. E. Keeting, E. M. Wilson, D. B. Lubahn, F. S. French, B. L. Riggs and T. C. Spelsberg (1989). "Identification of androgen receptors in normal human osteoblast-like cells." Proc Natl Acad Sci U S A 86(3): 854-7.

Compston, J. E. (1995). "Bone density: BMC, BMD, or corrected BMD? [editorial] " Bone 16(1): 5-7.

Concannon, P., K. J. Gogolin-Ewens, D. A. Hinds, B. Wapelhorst, V. A. Morrison, B. Stirling, M. Mitra, J. Farmer, S. R. Williams, N. J. Cox, G. I. Bell, N. Risch and R. S. Spielman (1998). "A second-generation screen of the human genome for susceptibility to insulin-dependent diabetes mellitus " Nat Genet 19(3): 292-6.

Consensus development conference V (1994). "Diagnosis, prophylaxis, and treatment of osteoporosis." Am J Med 90: 646-650.

Cooper, C. (1993). "Epidemiology and public health impact of osteoporosis." Balliere's Clinical Rheumatology 7(3): 1711-6.

Cooper, C., C. Fall, P. Egger, R. Hobbs, R. Eastell, D. Barker (1997). "Growth in infancy and bone mass in later life." Ann Rheum Dis 56(1): 17-21.

Cooper, C., E. J. Atkinson, S. J. Jacobsen, W. M. O'Fallon and L. J. Melton (1993). "Population-based study of survival after osteoporotic fractures." Am J Epidemiol 137(9): 1001-5.

Cooper, C., G. Campion and L. J. d. Melton (1992). "Hip fractures in the elderly: a world-wide projection." Osteoporos Int 2(6): 285-9.

Cooper, G. S. and D. M. Umbach (1996). "Are vitamin D receptor polymorphisms associated with bone mineral density? A meta-analysis " J Bone Miner Res 11(12): 1841-9.

Cordell, H. J., J. A. Todd, S. T. Bennett, Y. Kawaguchi and M. Farrall (1995). "Two-locus maximum lod score analysis of a multifactorial trait: joint consideration of IDDM2 and IDDM4 with IDDM1 in type 1 diabetes." Am J Hum Genet 57(4): 920-34.

Corder, E. H., A. M. Saunders, W. J. Strittmatter, D. E. Schmechel, P. C. Gaskell, G. W. Small, A. D. Roses, J. L. Haines and M. A. Pericak-Vance (1993). "Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families " Science 261(5123): 921-3.

Cox, N. J., M. Frigge, D. L. Nicolae, P. Concannon, C. L. Hanis, G. I. Bell and A. Kong (1999). "Loci on chromosomes 2 (NIDDM1) and 15 interact to increase susceptibility to diabetes in Mexican Americans." Nat Genet 21(2): 213-5.

Crabtree, N., M. Lunt, G. Holt, H. Kroger, H. Burger, S. Grazio, K. T. Khaw, R. S. Lorenc, J. Nijs, J. Stepan, J. A. Falch, T. Miazgowski, P. Raptou, H. A. Pols, J. Dequeker, S. Havelka, K. Hoszowski, I. Jajic, S. Czekalski, G. Lyritis, A. J. Silman and J. Reeve (2000). "Hip geometry, bone mineral distribution, and bone strength in European men and women: the EPOS study." Bone 27(1): 151-9.

Cummings, S., D. Black, M. Nevitt, W. Browner, J. Cauley, K. Ensrud, H. Genant, L. Palermo, J. Scott and T. Vogt (1993). "Bone density at various sites for prediction of hip fractures. The Study Osteoporosis Fractures Res Group." Lancet 341: 72-75.

Cummings, S. R., R. Marcus, L. Palermo, K. E. Ensrud and H. K. Genant (1994). "Does estimating volumetric bone density of the femoral neck improve the prediction of hip fracture? A prospective study. Study of Osteoporotic Fractures Research Group." J Bone Miner Res 9(9): 1429-32.

Cummings, S. R., M. C. Nevitt, W. S. Browner, K. Stone, K. M. Fox, K. E. Ensrud, J. Cauley, D. Black and T. M. Vogt (1995). "Risk factors for hip fracture in white women. Study of Osteoporotic Fractures Research Group." N Engl J Med 332(12): 767-73.

Curtis, D. (1996). "Genetic dissection of complex traits." Nat Genet 12(4): 356-8.

Curtis, D. (1997). "Use of siblings as controls in case-control association studies [published erratum appears in Ann Hum Genet 1998 Jan;62(Pt 1):89]." Ann Hum Genet 61(Pt 4): 319-33.

Curtis, D. and P. Sham (1995). "A note on the application of the transmission disequilibrium test when a parent is missing." Am J Hum Genet 56: 811-812.

Danielson, M. E., J. A. Cauley, C. E. Baker, A. B. Newman, J. S. Dorman, J. D. Towers and L. H. Kuller (1999). "Familial resemblance of bone mineral density (BMD) and calcaneal ultrasound attenuation: the BMD in mothers and daughters study." J Bone Miner Res 14(1): 102-10.

Danis, V. A., M. Millington, V. J. Hyland and D. Grennan (1995). "Cytokine production by normal human monocytes: inter-subject variation and relationship to an IL-1 receptor antagonist (IL-1Ra) gene polymorphism." Clin Exp Immunol 99(2): 303-10.

Davies, J. L., Y. Kawaguchi, S. T. Bennett, J. B. Copeman, H. J. Cordell, L. E. Pritchard, P. W. Reed, S. C. Gough, S. C. Jenkins, S. M. Palmer and et al. (1994). "A genome-wide search for human type 1 diabetes susceptibility genes " Nature 371(6493): 130-6.

Davis, S. and D. E. Weeks (1997). "Comparison of nonparametric statistics for detection of linkage in nuclear families: single-marker evaluation." Am J Hum Genet 61(6): 1431-44.

Dawson-Hughes, B., S. S. Harris, E. A. Krall and G. E. Dallal (1997). "Effect of calcium and vitamin D supplementation on bone density in men and women 65 years of age or older " N Engl J Med 337(10): 670-6.

de Andrade, M., T. J. Thiel, L. Yu and C. I. Amos (1997). "Assessing linkage on chromosome 5 using components of variance approach: univariate versus multivariate." Genet Epidemiol 14(6): 773-8.

De Laet, C. E., B. A. Van Hout, H. Burger, A. E. Weel, A. Hofman and H. A. Pols (1998). "Hip fracture prediction in elderly men and women: validation in the Rotterdam study." J Bone Miner Res 13(10): 1587-93.

Dean, V., E. E. Hobson, R. M. Aspden, S. P. Robins and S. H. Ralston (1998). "Relationship between COL1A1 Sp1 alleles, gene transcription, collagen production and bone strength." Bone 23(5): S161.

Deftos, L., B. Roos and E. Oates (1999). Calcitonin. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. M. Favus. Philadelphia, Lippincott, Williams & Wilkins.

Delany, A., J. Pash and E. Canalis (1994). "Cellular and clinical perspectives on skeletal insulin-like growth factor 1." J Cell Biochem 55: 328-333.

Deng, H. W., W. M. Chen, T. Conway, Y. Zhou, K. M. Davies, M. R. Stegman, H. Deng and R. R. Recker (2000). "Determination of bone mineral density of the hip and spine in human pedigrees by genetic and life-style factors." Genet Epidemiol 19(2): 160-77.

Deng, H. W., J. Li, J. L. Li, R. Dowd, K. M. Davies, M. Johnson, G. Gong, H. Deng and R. R. Recker (2000). "Association of estrogen receptor-alpha genotypes with body mass index in normal healthy postmenopausal Caucasian women." J Clin Endocrinol Metab 85(8): 2748-51.

Deng, H. W., M. R. Stegman, K. M. Davies, T. Conway and R. R. Recker (1999). "Genetic determination of variation and covariation of peak bone mass at the hip and spine." J Clin Densitom 2(3): 251-63.

Devoto, M., K. Shimoya, J. Caminis, J. Ott, A. Tenenhouse, M. Whyte, L. Sereda, S. Hall, E. Considine, C. Williams, G. Tromp, H. Kuivaniemi, L. Ala-Kokko, D. Prockop and L. Spotila (1998). "First-stage autosomal genome screen in extended pedigrees suggests genes predisposing to low bone mineral density on chromosomes 1p, 2p and 4q." Eur J Hum Genet 6: 151-157.

Dobson-Stone, C., R. D. Cox, L. Lonie, L. Southam, M. Fraser, C. Wise, F. Bernier, S. Hodgson, D. E. Porter, A. H. Simpson and A. P. Monaco (2000). "Comparison of fluorescent single-strand conformation polymorphism analysis and denaturing high-performance liquid chromatography for detection of EXT1 and EXT2 mutations in hereditary multiple exostoses." Eur J Hum Genet 8(1): 24-32.

Dohi, Y., M. Iki, H. Ohgushi, S. Gojo, S. Tabata, E. Kajita, H. Nishino and K. Yonemasu (1998). "A novel polymorphism in the promoter region for the human osteocalcin gene: the possibility of a correlation with bone mineral density in postmenopausal Japanese women." J Bone Miner Res 13(10): 1633-9.

Dougall, W. C., M. Glaccum, K. Charrier, K. Rohrbach, K. Brasel, T. De Smedt, E. Daro, J. Smith, M. E. Tometsko, C. R. Maliszewski, A. Armstrong, V. Shen, S. Bain, D. Cosman, D. Anderson, P. J. Morrissey, J. J. Peschon and J. Schuh (1999). "RANK is essential for osteoclast and lymph node development." Genes Dev 13(18): 2412-24.

Douglas, J. A., M. Boehnke and K. Lange (2000). "A multipoint method for detecting genotyping errors and mutations in sibling-pair linkage data." Am J Hum Genet 66(4): 1287-97.

Duan, Y., V. De Luca and E. Seeman (1999). "Parathyroid hormone deficiency and excess: similar effects on trabecular bone but differing effects on cortical bone." J Clin Endocrinol Metab 84(2): 718-22.

Ducy, P., C. Desbois and B. e. a. Boyce (1996). "Increased bone formation in osteocalcin-deficient mice." Nature 382: 448-451.

Dudbridge, F., B. P. Koeleman, J. A. Todd and D. G. Clayton (2000). "Unbiased Application of the Transmission/Disequilibrium Test to Multilocus Haplotypes." Am J Hum Genet 66(6): 2009-2012.

Duncan, E. and J. A. Wass (1999). "Investigation protocol: acromegaly and its investigation." Clin Endocrinol (Oxf) 50(3): 285-93.

Eastell, R., I. T. Boyle, J. Compston, C. Cooper, I. Fogelman, R. M. Francis, D. J. Hosking, D. W. Purdie, S. Ralston, J. Reeve, D. M. Reid, R. G. Russell and J. C. Stevenson (1998). "Management of male osteoporosis: report of the UK Consensus Group." QJM 91(2): 71-92.

Eastell, R. E., M. S. Calvo, M. F. Burritt, K. P. Offord, R.G. Russell, B. L. Riggs (1992) "Abnormalities in circadian pattersn of bone resoption and renal calcium conservation in type 1 osteoporosis." J Clin Endocrinol Metab 74(3): 487-94.

Eaves, I. A., T. R. Merriman, R. A. Barber, S. Nutland, E. Tuomilehto-Wolf, J. Tuomilehto, F. Cucca and J. A. Todd (2000). "The genetically isolated populations of Finland and sardinia may not be a panacea for linkage disequilibrium mapping of common disease genes " Nat Genet 25(3): 320-3.

Ebeling, P. R., J. D. Jones, W. M. O'Fallon, C. H. Janes and B. L. Riggs (1993). "Short-term effects of recombinant human insulin-like growth factor I on bone turnover in normal women." J Clin Endocrinol Metab 77(5): 1384-7.

Eccleshall, T. R., P. Garnero, C. Gross, P. D. Delmas and D. Feldman (1998). "Lack of correlation between start codon polymorphism of the vitamin D receptor gene and bone mineral density in premenopausal French women: the OFELY study." J Bone Miner Res 13(1): 31-5.

Eisenberg, S., R. Evans, W. Arend, E. Verdeerber, M. Breser, C. Hannum and R. Thompson (1990). "Primary structure and functional expression from complementary DNA of a human interleukin-1 receptor antagonist." Nature 343: 341-346.

Eisman, J. A. (1995). "Vitamin D receptor gene alleles and osteoporosis: an affirmative view." J Bone Miner Res 10(9): 1289-93.

Elston, R. (1997). "1996 William Allan Award Address. Algorithms and inferences: the challenge of multifactorial diseases." Am J Hum Genet 60: 255-262.

Elston, R. C. (1998). "Linkage and association." Genet Epidemiol 15(6): 565-76.

Ensrud, K. E., K. Stone, J. A. Cauley, C. White, J. M. Zmuda, T. V. Nguyen, J. A. Eisman and S. R. Cummings (1999). "Vitamin D receptor gene polymorphisms and the risk of fractures in older women. For the Study of Osteoporotic Fractures Research Group." J Bone Miner Res 14(10): 1637-45.

Eriksen, E. F., D. S. Colvard, N. J. Berg, M. L. Graham, K. G. Mann, T. C. Spelsberg and B. L. Riggs (1988). "Evidence of estrogen receptors in normal human osteoblast-like cells." Science 241(4861): 84-6.

Erlebacher, A., E. H. Filvaroff, J. Q. Ye and R. Derynck (1998). "Osteoblastic responses to TGF-beta during bone remodeling." Mol Biol Cell 9(7): 1903-18.

Escary, J. L., L. Lonie, R. D. Cox, J. M. Walter and M. Lathrop (1999). "Screening strategy for mutation detection using denaturing high-performance liquid chromatography (DHPLC)." International Biotechnology Laboratory April 1999: 19.

Evans, R. A., G. M. Marel, E. K. Lancaster, S. Kos, M. Evans and S. Y. Wong (1988). "Bone mass is low in relatives of osteoporotic patients." Ann Intern Med 109(11): 870-3.

Fagiolo, U., A. Cossarizza, E. Scala, E. Fanales-Belasio, C. Ortolani, E. Cozzi, D. Monti, C. Franceschi and R. Paganelli (1993). "Increased cytokine production in mononuclear cells of healthy elderly people." Eur J Immunol 23(9): 2375-8.

Falahati, A., B. Riggs, E. Atkinson, W. O'Fallon and S. Khosla (2000). "Estrogen, but not testosterone, prevents the increase in bone resorption following induction of hypogonadism and aromatase inhibition in normal elderly men." J Bone Miner Res 15(Suppl 1): S148.

Falconer, D. (1964). Introduction to Quantitative Genetics. London, Oliver and Boyd.

Falk, C. and P. Rubinstein (1987). "Haplotype relative risks: an easy reliable way to construct a proper control sample for risk calculations." Ann Hum Genet 51: 227-233.

Farfel, Z., H. R. Bourne and T. Iiri (1999). "The expanding spectrum of G protein diseases." N Engl J Med 340(13): 1012-20.

Faulkner, K. and E. Orwoll (2000). "Use of the WHO Criterion in Men: is –2.5 the Right Number?" J Bone Miner Res 15(Suppl 1): S169.

Faulkner, K. G., L. A. Roberts and M. R. McClung (1996). "Discrepancies in normative data between Lunar and Hologic DXA systems." Osteoporos Int 6(6): 432-6.

Faulkner, R. A., R. G. McCulloch, S. L. Fyke, W. E. De Coteau, H. A. McKay, D. A. Bailey, C. S. Houston and A. A. Wilkinson (1995). "Comparison of areal and estimated volumetric bone mineral density values between older men and women." Osteoporos Int 5(4): 271-5.

Felix, R., M. G. Cecchini and H. Fleisch (1990). "Macrophage colony stimulating factor restores in vivo bone resorption in the op/op osteopetrotic mouse." Endocrinology 127(5): 2592-4.

Felson, D. T., Y. Zhang, M. T. Hannan and J. J. Anderson (1993). "Effects of weight and body mass index on bone mineral density in men and women: the Framingham study." J Bone Miner Res 8(5): 567-73.

Ferrari, S., R. Rizzoli, T. Chevalley, D. Slosman, J. A. Eisman and J. P. Bonjour (1995). "Vitamin-D-receptor-gene polymorphisms and change in lumbar-spine bone mineral density." Lancet 345(8947): 423-4.

Ferrari, S., R. Rizzoli, D. Manen, D. Slosman and J. P. Bonjour (1998). "Vitamin D receptor gene start codon polymorphisms (FokI) and bone mineral density: interaction with age, dietary calcium, and 3'-end region polymorphisms." J Bone Miner Res 13(6): 925-30.

Ferrari, S. L., R. Rizzoli, D. O. Slosman and J. P. Bonjour (1998). "Do dietary calcium and age explain the controversy surrounding the relationship between bone mineral density and vitamin D receptor gene polymorphisms?" J Bone Miner Res 13(3): 363-70.

Firooznia, H., M. Rafii, C. Golimbu, M. S. Schwartz and P. Ort (1986). "Trabecular mineral content of the spine in women with hip fracture: CT measurement." Radiology 159(3): 737-40.

Fisher, R. (1918). "The correlation between relatives on the supposition of Mendelian inheritance." Translations of the Royal Society, Edinburgh. 52: 399-433.

Fisher, S. E., A. J. Marlow, J. Lamb, E. Maestrini, D. F. Williams, A. J. Richardson, D. E. Weeks, J. F. Stein and A. P. Monaco (1999). "A quantitative-trait locus on chromosome 6p influences different aspects of developmental dyslexia." Am J Hum Genet 64(1): 146-56.

Fishman, D., G. Faulds, R. Jeffery, V. Mohamed-Ali, J. S. Yudkin, S. Humphries and P. Woo (1998). "The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis." J Clin Invest 102(7): 1369-76.

Flicker, L., J. L. Hopper, L. Rodgers, B. Kaymakci, R. M. Green and J. D. Wark (1995). "Bone density determinants in elderly women: a twin study." J Bone Miner Res 10(11): 1607-13.

Forsen, L., A. Sogaard, H. Meyer, T.-H. Edna and B. Kopjar (1999). "Survival after hip fracture: short- and long-term excess mortality according to age and gender." Osteoporosis Int 10: 73-78.

Fox, K. M., S. R. Cummings, K. Powell-Threets and K. Stone (1998). "Family history and risk of osteoporotic fracture." Osteoporosis Int 8: 557-562.

Francis, R. M., F. Harrington, E. Turner, S. S. Papiha and H. K. Datta (1997). "Vitamin D receptor gene polymorphism in men and its effect on bone density and calcium absorption." Clin Endocrinol Oxf 46(1): 83-6.

Fraser, W. D., F. C. Logue, J. P. Christie, S. J. Gallacher, D. Cameron, D. S. O'Reilly, G. H. Beastell, I.T. Boyle (1998) "Alteration in the circadian rhythm of intact parathyroid hormone and serum phosphate in women with established postmenopausal osteoporosis." Osteoporosis Int 8(2): 121-6.

Fulker, D. W., S. S. Cherny, P. C. Sham and J. K. Hewitt (1999). "Combined linkage and association sib-pair analysis for quantitative traits." Am J Hum Genet 64(1): 259-67.

Fuller, K., B. Wong, S. Fox, Y. Choi and T. J. Chambers (1998). "TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts." J Exp Med 188(5): 997-1001.

Gale, C.R., C. N. Martyn, S, Kellingray, R. Eastell, and C. Cooper (2001). "Intrauterine programming of adult body composition." J Clin Endocrinol Metab 86(1): 267-72.

Gao, Y. H., T. Shinki, T. Yuasa, H. Kataoka-Enomoto, T. Komori, T. Suda and A. Yamaguchi (1998). "Potential role of cbfa1, an essential transcriptional factor for osteoblast differentiation, in osteoclastogenesis: regulation of mRNA expression of osteoclast differentiation factor (ODF)." Biochem Biophys Res Commun 252(3): 697-702.

Garcia-Giralt, N., A. Enjuanes, A. Bay Jensen, X. Nogues, S. Quintana, J. Puig, L. Mellibovsky, A. Diez, D. Grinberg and S. Balcelles (2000). "A new SNP in the upstream regulatory region of COL1A1 is associated with BMD in perimenopausal women from Spain." J Bone Miner Res 15(Suppl 1): S490.

Garnero, P., N. K. Arden, G. Griffiths, P. D. Delmas and T. D. Spector (1996). "Genetic influence on bone turnover in postmenopausal twins." J Clin Endocrinol Metab 81(1): 140-6.

Garnero, P., O. Borel, S. F. Grant, S. H. Ralston and P. D. Delmas (1998). "Collagen Ialpha1 Sp1 polymorphism, bone mass, and bone turnover in healthy French premenopausal women: the OFELY study." J Bone Miner Res 13(5): 813-7.

Garnero, P., O. Borel, E. Sornay Rendu, M. E. Arlot and P. D. Delmas (1996). "Vitamin D receptor gene polymorphisms are not related to bone turnover, rate of bone loss, and bone mass in postmenopausal women: the OFELY Study." J-Bone-Miner-Res 11(6): 827-34.

Garnero, P., O. Borel, E. Sornay Rendu and P. D. Delmas (1995). "Vitamin D receptor gene polymorphisms do not predict bone turnover and bone mass in healthy premenopausal women." J-Bone-Miner-Res 10(9): 1283-8.

Garnero, P., E. Hausherr, M. C. Chapuy, C. Marcelli, H. Grandjean, C. Muller, C. Cormier, G. Breart, P. J. Meunier and P. D. Delmas (1996). "Markers of bone resorption predict hip fracture in elderly women: the EPIDOS Prospective Study." J Bone Miner Res 11(10): 1531-8.

Garrett, J. E., I. V. Capuano, L. G. Hammerland, B. C. Hung, E. M. Brown, S. C. Hebert, E. F. Nemeth and F. Fuller (1995). "Molecular cloning and functional expression of human parathyroid calcium receptor cDNAs." J Biol Chem 270(21): 12919-25.

Geiser, A. G., Q. Q. Zeng, M. Sato, L. M. Helvering, T. Hirano and C. H. Turner (1998). "Decreased bone mass and bone elasticity in mice lacking the transforming growth factor-beta1 gene." Bone 23(2): 87-93.

Genant, H., C. Cooper, G. Poor, I. Reid, G. Ehrlich and J. Kanis (1999). "Interim Report and Recommendations of the Wolrd Health Organization Task-Force for Osteoporosis." Osteoporosis Int 10: 259-64.

Genant, H. K., K. Engelke, T. Fuerst, C. C. Gluer, S. Grampp, S. T. Harris, M. Jergas, T. Lang, Y. Lu, S. Majumdar, A. Mathur and M. Takada (1996). "Noninvasive assessment of bone mineral and structure: state of the art." J Bone Miner Res 11(6): 707-30.

Gennari, L., L. Becherini, L. Masi, R. Mansani, S. Gonnelli, C. Cepollaro, S. Martini, A. Montagnani, G. Lentini, A. M. Becorpi and M. L. Brandi (1998). "Vitamin D and estrogen receptor allelic variants in Italian postmenopausal women: evidence of multiple gene contribution to bone mineral density." J Clin Endocrinol Metab 83(3): 939-44.

Gerstenfeld, L. C. (1999). "Osteopontin in skeletal tissue homeostasis: An emerging picture of the autocrine/paracrine functions of the extracellular matrix [editorial; comment]." J Bone Miner Res 14(6): 850-5.

Gilsanz, V., E. Shultz and L. Loro (1993). "Smaller cross-sectional area of intact vertebral bodies in women with vertebral fractures." J Bone Miner Res 8(Suppl 1): S326.

Girasole, G., R. L. Jilka, G. Passeri, S. Boswell, G. Boder, D. C. Williams and S. C. Manolagas (1992). "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens." J Clin Invest 89(3): 883-91.

Girasole, G., G. Passeri, R. L. Jilka and S. C. Manolagas (1994). "Interleukin-11: a new cytokine critical for osteoclast development." J Clin Invest 93(4): 1516-24.

Girasole, G., M. Pedrazzoni, N. Giuliani, G. Passeri and M. Passeri (1995). "Increased serum soluble interleukin-6 receptor levles are induced by ovariectomy, prevented by estrogen replacement, and reversed by alendronate administration." J Bone Miner Res 10: A86.

Glimcher, M. (1998). The nature of the mineral phase in bone: biological and clinical implications. Metabolic gone disease and clinically related disorders. L. Avioli and K. SM. San Diego: 23-51.

Goltzman, D. (1999). "Interactions of PTH and PTHrP with the PTH/PTHrP receptor and with downstream signaling pathways: exceptions that provide the rules [editorial; comment] " J Bone Miner Res 14(2): 173-7.

Gong, G., M. L. Johnson, M. J. Barger-Lux and R. P. Heaney (1999). "Association of bone dimensions with a parathyroid hormone gene polymorphism in women." Osteoporos Int 9(4): 307-11.

Gong, Y., M. Vikkula, L. Boon, J. Liu, P. Beighton, R. Ramesar, L. Peltonen, H. Somer, T. Hirose, B. Dallapiccola, A. De Paepe, W. Swoboda, B. Zabel, A. Superti-Furga, B. Steinmann, H. G. Brunner, A. Jans, R. G. Boles, W. Adkins, M. J. van den Boogaard, B. R. Olsen and M. L. Warman (1996). "Osteoporosis-pseudoglioma syndrome, a disorder affecting skeletal strength and vision, is assigned to chromosome region 11q12-13." Am J Hum Genet 59(1): 146-51.

Goodman-Gruen, D. and E. Barrett-Connor (1997). "Epidemiology of insulin-like growth factor-I in elderly men and women. The Rancho Bernardo Study [published erratum appears in Am J Epidemiol 1997 Aug 15;146(4):357] " Am J Epidemiol 145(11): 970-6.

Gorn, A. H., H. Y. Lin, M. Yamin, P. E. Auron, M. R. Flannery, D. R. Tapp, C. A. Manning, H. F. Lodish, S. M. Krane and S. R. Goldring (1992). "Cloning, characterization, and expression of a human calcitonin receptor from an ovarian carcinoma cell line." J Clin Invest 90(5): 1726-35.

Gough, A., P. Sambrook, J. Devlin, J. Lilley, A. Huisoon, J. Betteridge, J. Franklyn, T. Nguyen, N. Morrison, J. Eisman and P. Emery (1998). "Effect of vitamin D receptor gene alleles on bone loss in early rheumatoid arthritis." J Rheumatol 25(5): 864-8.

Grainger, D. J., K. Heathcote, M. Chiano, H. Snieder, P. R. Kemp, J. C. Metcalfe, N. D. Carter and T. D. Spector (1999). "Genetic control of the circulating concentration of transforming growth factor type beta1." Hum Mol Genet 8(1): 93-7.

Grant, S., D. Reid, G. Blake, R. Herd, I. Fogelman and S. Ralston (1996). "Reduced bone density and osteoporosis associated with a polymorphic Sp1 binding site in the collagen type I alpha 1 gene." Nat Genet 14(2): 203-205.

Greendale, G. A., S. Edelstein and E. Barrett-Connor (1997). "Endogenous sex steroids and bone mineral density in older women and men: the Rancho Bernardo Study." J Bone Miner Res 12(11): 1833-43.

Gross, C., T. R. Eccleshall, P. J. Malloy, M. L. Villa, R. Marcus and D. Feldman (1996). "The presence of a polymorphism at the translation initiation site of the vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican-American women." J Bone Miner Res 11(12): 1850-5.

Gross, C., A. V. Krishnan, P. J. Malloy, T. R. Eccleshall, X. Y. Zhao and D. Feldman (1998). "The vitamin D receptor gene start codon polymorphism: a functional analysis of FokI variants." J Bone Miner Res 13(11): 1691-9.

Gu, C. and D. Rao (1997). "A Linkage Strategy for Detection of Human Quantitative-trai Loci. II Optimization of study Designs Based on Extreme Sib Pairs and Generalized Relative Risk Ratios." Am J Hum Genet 61: 211-222.

Gu, C. and D. C. Rao (1997). "A linkage strategy for detection of human quantitative-trait loci. I. Generalized relative risk ratios and power of sib pairs with extreme trait values." Am J Hum Genet 61(1): 200-10.

Gu, C., A. Todorov and D. C. Rao (1996). "Combining extremely concordant sibpairs with extremely discordant sibpairs provides a cost effective way to linkage analysis of quantitative trait loci." Genet Epidemiol 13(6): 513-33.

Gu, C., A. A. Todorov and D. C. Rao (1997). "Genome screening using extremely discordant and extremely concordant sib pairs." Genet Epidemiol 14(6): 791-6.

Gueguen, R., P. Jouanny, F. Guillemin, C. Kuntz, J. Pourel and G. Siest (1995). "Segregation analysis and variance components analysis of bone mineral density in healthy families." J Bone Miner Res 10(12): 2017-22.

Gundberg, C. M. (2000). Biochemical markers of bone formation. Osteoporosis and Metabolic Bone Diseases. C. Rosen. Philadelphia, W B Saunders. 20: 489-502.

Hall, J. M., L. Friedman, C. Guenther, M. K. Lee, J. L. Weber, D. M. Black and M. C. King (1992). "Closing in on a breast cancer gene on chromosome 17q." Am J Hum Genet 50(6): 1235-42.

Halushka, M. K., J. B. Fan, K. Bentley, L. Hsie, N. Shen, A. Weder, R. Cooper, R. Lipshutz and A. Chakravarti (1999). "Patterns of single-nucleotide polymorphisms in candidate genes for blood-pressure homeostasis." Nat Genet 22(3): 239-47.

Han, K. O., I. G. Moon, C. S. Hwang, J. T. Choi, H. K. Yoon, H. K. Min and I. K. Han (1999). "Lack of an intronic Sp1 binding-site polymorphism at the collagen type I alpha1 gene in healthy Korean women." Bone 24(2): 135-7.

Han, K. O., I. G. Moon, Y. S. Kang, H. Y. Chung, H. K. Min and I. K. Han (1997). "Nonassociation of estrogen receptor genotypes with bone mineral density and estrogen responsiveness to hormone replacement therapy in Korean postmenopausal women." J Clin Endocrinol Metab 82(4): 991-5.

Handford, P., A. K. Downing, Z. Rao, D. R. Hewett, B. C. Sykes and C. M. Kielty (1995). "The calcium binding properties and molecular organization of epidermal growth factor-like domains in human fibrillin-1." J Biol Chem 270(12): 6751-6.

Hanis, C. L., E. Boerwinkle, R. Chakraborty, D. L. Ellsworth, P. Concannon, B. Stirling, V. A. Morrison, B. Wapelhorst, R. S. Spielman, K. J. Gogolin-Ewens, J. M. Shepard, S. R. Williams, N. Risch, D. Hinds, N. Iwasaki, M. Ogata, Y. Omori, C. Petzold, H. Rietzch, H. E. Schroder, J. Schulze, N. J. Cox, S. Menzel, V. V. Boriraj, X. Chen and et al. (1996). "A genome-wide search for human non-insulin-dependent (type 2) diabetes genes reveals a major susceptibility locus on chromosome 2 " Nat Genet 13(2): 161-6.

Hannan, M. T., D. T. Felson, B. Dawson-Hughes, K. L. Tucker, L. A. Cupples, P. W. Wilson and D. P. Kiel (2000). "Risk factors for longitudinal bone loss in elderly men and women: the Framingham Osteoporosis Study." J Bone Miner Res 15(4): 710-20.

Hansen, M. A., C. Hassager, S. B. Jensen and C. Christiansen (1992). "Is heritability a risk factor for postmenopausal osteoporosis?" J Bone Miner Res 7(9): 1037-43.

Harris, M., T. V. Nguyen, G. M. Howard, P. J. Kelly and J. A. Eisman (1998). "Genetic and environmental correlations between bone formation and bone mineral density: a twin study." Bone 22(2): 141-5.

Harris, S. S., T. R. Eccleshall, C. Gross, B. Dawson Hughes and D. Feldman (1997). "The vitamin D receptor start codon polymorphism (FokI) and bone mineral density in premenopausal American black and white women." J Bone Miner Res 12(7): 1043-8.

Harris, S. S., M. S. Patel, D. E. Cole and B. Dawson-Hughes (2000). "Associations of the collagen type Ialpha1 Sp1 polymorphism with five-year rates of bone loss in older adults." Calcif Tissue Int 66(4): 268-71.

Haseman, J. and R. Elston (1972). "The investigation of linkage between a quantitative trait and a marker locus." Behav Genet 2: 3-19.

Hashimoto, L., C. Habita, J. P. Beressi, M. Delepine, C. Besse, A. Cambon-Thomsen, I. Deschamps, J. I. Rotter, S. Djoulah, M. R. James and et al. (1994). "Genetic mapping of a susceptibility locus for insulin-dependent diabetes mellitus on chromosome 11q " Nature 371(6493): 161-4.

Hasstedt, S. (1994). Pedigree Analysis Package. Salt Lake City, Utah, Department of Human Genetics, University of Utah.

Hata, R., H. Hori, Y. Nagai, S. Tanaka, M. Kondo, M. Hiramatsu, N. Utsumi and M. Kumegawa (1984). "Selective inhibition of type I collagen synthesis in osteoblastic cells by epidermal growth factor." Endocrinology 115(3): 867-76.

Hattersley, G., J. Kerby and T. Chambers (1991). "Identification of osteoclast precursors in multilineage hemopoietic colonies." Endocrinology 128: 259-62.

Haughton, M. A., A. S. Gunnell, S. F. Grant, M. A. Brown and J. A. Eisman (1998). "Linkage studies of the COL1A1 and VDR loci in the control of bone mineral density." J Bone Miner Res 13: S370.

Haussler, M., G. Whitfield, C. Haussler, J.-C. Hsieh, P. Thompson, S. Selznick, C. Dominguez and P. Jurutka (1998). "The Nuclear Vitamin D Receptor: Biological and Molecular Regulatory Properties Revealed." J Bone Miner Res 13(3): 325-349.

Heaney, C., H. Shalev, K. Elbedour, R. Carmi, J. B. Staack, V. C. Sheffield and D. R. Beier (1998). "Human autosomal recessive osteopetrosis maps to 11q13, a position predicted by comparative mapping of the murine osteosclerosis (oc) mutation." Hum Mol Genet 7(9): 1407-10.

Heegaard, A., H. L. Jorgensen, A. W. Vestergaard, C. Hassager and S. H. Ralston (2000). "Lack of influence of collagen type Ialpha1 Sp1 binding site polymorphism on the rate of bone loss in a cohort of postmenopausal danish women followed for 18 years." Calcif Tissue Int 66(6): 409-13.

Henry, Y. M. and R. Eastell (2000). "Ethnic and gender differences in bone mineral density and bone turnover in young adults: effect of bone size." Osteoporos Int 11(6): 512-7.

Heymann, D. and A. V. Rousselle (2000). "gp130 Cytokine family and bone cells " Cytokine 12(10): 1455-68.

Hodge, S. (1984). "The information contained in multiple sibling pairs." Genet. Epidemiol. 1: 109-164.

Hodge, S. and R. Elston (1994). "Lods, wrods and mods,: the interpretation of lod scores calculated under different models." Genetic Epidemiology 11: 329-42.

Hofbauer, L. C., C. R. Dunstan, T. C. Spelsberg, B. L. Riggs and S. Khosla (1998). "Osteoprotegerin production by human osteoblast lineage cells is stimulated by vitamin D, bone morphogenetic protein-2, and cytokines." Biochem Biophys Res Commun 250(3): 776-81.

Hofbauer, L. C., S. Khosla, C. R. Dunstan, D. L. Lacey, W. J. Boyle and B. L. Riggs (2000). "The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption." J Bone Miner Res 15(1): 2-12.

Horikawa, Y., N. Oda, N. J. Cox, X. Li, M. Orho-Melander, M. Hara, Y. Hinokio, T. H. Lindner, H. Mashima, P. E. Schwarz, L. del Bosque-Plata, Y. Oda, I. Yoshiuchi, S. Colilla, K. S. Polonsky, S. Wei, P. Concannon, N. Iwasaki, J. Schulze, L. J. Baier, C. Bogardus, L. Groop, E. Boerwinkle, C. L. Hanis and G. I. Bell (2000). "Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus " Nat Genet 26(2): 163-75.

Hosoi, T., M. Miyao, S. Inoue, S. Hoshino, M. Shiraki, H. Orimo and Y. Ouchi (1999). "Association study of parathyroid hormone gene polymorphism and bone mineral density in Japanese postmenopausal women." Calcif Tissue Int 64(3): 205-8.

Howard, G. M., T. V. Nguyen, M. Harris, P. J. Kelly and J. A. Eisman (1998). "Genetic and environmental contributions to the association between quantitative ultrasound and bone mineral density measurements: a twin study." J Bone Miner Res 13(8): 1318-27.

Hoyland, J. A., A. P. Mee, P. Baird, I. P. Braidman, E. B. Mawer and A. J. Freemont (1997). "Demonstration of estrogen receptor mRNA in bone using in situ reverse-transcriptase polymerase chain reaction." Bone 20(2): 87-92.

Hsu, H., D. L. Lacey, C. R. Dunstan, I. Solovyev, A. Colombero, E. Timms, H. L. Tan, G. Elliott, M. J. Kelley, I. Sarosi, L. Wang, X. Z. Xia, R. Elliott, L. Chiu, T. Black, S. Scully, C. Capparelli, S. Morony, G. Shimamoto, M. B. Bass and W. J. Boyle (1999). "Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand." Proc Natl Acad Sci U S A 96(7): 3540-5.

Hughes, A. E., S. H. Ralston, J. Marken, C. Bell, H. MacPherson, R. G. Wallace, W. van Hul, M. P. Whyte, K. Nakatsuka, L. Hovy and D. M. Anderson (2000). "Mutations in TNFRSF11A, affecting the signal peptide of RANK, cause familial expansile osteolysis." Nat Genet 24(1): 45-8.

Hunter, D., M. De Lange, H. Snieder, A. J. MacGregor, R. Swaminathan, R. V. Thakker and T. D. Spector (2001). "Genetic contribution to bone metabolism, calcium excretion, and vitamin D and parathyroid hormone regulation." J Bone Miner Res 16(2): 371-8.

Hustmyer, F. G., G. Liu, C. C. Johnston, J. Christian and M. Peacock (1999). "Polymorphism at an Sp1 binding site of COL1A1 and bone mineral density in premenopausal female twins and elderly fracture patients." Osteoporos Int 9(4): 346-50.

Hustmyer, F. G., M. Peacock, S. Hui, C. C. Johnston and J. Christian (1994). "Bone mineral density in relation to polymorphism at the vitamin D receptor gene locus." J Clin Invest 94(5): 2130-4.

Hustmyer, F. G., E. Schipani and M. Peacock (1993). "BsmI polymorphism at the parathyroid hormone receptor locus (PTHR) in three populations." Hum Mol Genet 2(8): 1330.

Ismail, A. A., T. W. O'Neill, C. Cooper, J. D. Finn, A. K. Bhalla, J. B. Cannata, P. Delmas, J. A. Falch, B. Felsch, K. Hoszowski, O. Johnell, J. B. Diaz-Lopez, A. Lopez Vaz, F. Marchand, H. Raspe, D. M. Reid, C. Todd, K. Weber, A. Woolf, J. Reeve and A. J. Silman (1998). "Mortality associated with vertebral deformity in men and women: results from the European Prospective Osteoporosis Study (EPOS)." Osteoporos Int 8(3): 291-7.

Iyengar, S., F. Calafell and K. K. Kidd (1997). "Detection of major genes underlying several quantitative traits associated with a common disease using different ascertainment schemes." Genet Epidemiol 14(6): 809-14.

Jacobsen, S., J. Goldberg and T. Miles (1992). "Race and sex differences in mortality following fracture of the hip." Am J Public Health 82: 1147-1150.

Jaenisch, R. (1997). "DNA methylation and imprinting: why bother?" Trends in Genetics. 13(8): 323-329.

Jarvik, G. P. (1998). "Complex segregation analyses: uses and limitations [comment]." Am J Hum Genet 63(4): 942-6.

Jawaheer, D., A. MacGregor, P. Gregersen, A. Silman and W. Ollier (1996). "Unexpected HLA haplotype sharing in dizygotic twin pairs discordant for rheumatoid arthritis." J Med Genet 1996(33): 1015-1018.

Jeanpierre, M. (1987). "A rapid method for the purification of DNA from blood." Nucleic-Acids-Res 15(22): 9611.

Jilka, R. L. (1998). "Cytokines, bone remodeling, and estrogen deficiency: a 1998 update." Bone 23(2): 75-81.

Jilka, R. L., G. Hangoc, G. Girasole, G. Passeri, D. C. Williams, J. S. Abrams, B. Boyce, H. Broxmeyer and S. C. Manolagas (1992). "Increased osteoclast development after estrogen loss: mediation by interleukin-6." Science 257(5066): 88-91.

Jilka, R. L., R. S. Weinstein, K. Takahashi, A. M. Parfitt and S. C. Manolagas (1996). "Linkage of decreased bone mass with impaired osteoblastogenesis in a murine model of accelerated senescence." J Clin Invest 97(7): 1732-40.

Jin, L., S. L. Briggs, S. Chandrasekhar, N. Y. Chirgadze, D. K. Clawson, R. W. Schevitz, D. L. Smiley, A. H. Tashjian and F. Zhang (2000). "Crystal structure of human parathyroid hormone 1-34 at 0.9-A resolution." J Biol Chem 275(35): 27238-44.

Jobert, A. S., P. Zhang, A. Couvineau, J. Bonaventure, J. Roume, M. Le Merrer and C. Silve (1998). "Absence of functional receptors for parathyroid hormone and parathyroid hormone-related peptide in Blomstrand chondrodysplasia." J Clin Invest 102(1): 34-40.

Johnson, M., G. Gong, D. Kimmel, M. Berger-Lux, R. Heaney, J. Lappe and R. Recker (1995). "Lack of correlation between parathyroid hormone gene alleles and skeletal phenotype." J Bone Miner Res 10(Suppl 1): s367.

Johnson, M. L., G. Gong, W. Kimberling, S. M. Recker, D. B. Kimmel and R. B. Recker (1997). "Linkage of a gene causing high bone mass to human chromosome 11 (11q12-13)." Am J Hum Genet 60(6): 1326-32.

Jones, A. C., J. Austin, N. Hansen, B. Hoogendoorn, P. J. Oefner, J. P. Cheadle and M. C. O'Donovan (1999). "Optimal temperature selection for mutation detection by denaturing HPLC and comparison to single-stranded conformation polymorphism and heteroduplex analysis." Clin Chem 45(8 Pt 1): 1133-40.

Jones, G. and T. Nguyen (2000). "Associations Betwen Maternal Peak Bone Mass and Bone Mass in Prepubertal Male and Female Children." J Bone Miner Res 15(10): 1998-2004.

Jones, G., T. Nguyen, P. N. Sambrook, P. J. Kelly, C. Gilbert and J. A. Eisman (1994). "Symptomatic fracture incidence in elderly men and women: the Dubbo Osteoporosis Epidemiology Study (DOES)." Osteoporos Int 4(5): 277-82.

Joosten, L. A., E. Lubberts, M. M. Helsen, T. Saxne, C. J. Coenen-De Roo, D. Heinegard and W. B. van Den Berg (1999). "Protection against cartilage and bone destruction by systemic interleukin-4 treatment in established murine type II collagen-induced arthritis." Arthritis Res 1(1): 81-91.

Jorgensen, H. L., J. Scholler, J. C. Sand, M. Bjuring, C. Hassager and C. Christiansen (1996). "Relation of common allelic variation at vitamin D receptor locus to bone mineral density and postmenopausal bone loss: cross sectional and longitudinal population study." Bmj 313(7057): 586-90.

Jouanny, P., F. Guillemin, C. Kuntz, C. Jeandel and J. Pourel (1995). "Environmental and genetic factors affecting bone mass. Similarity of bone density among members of healthy families." Arthritis Rheum 38(1): 61-7.

Jüppner, H. (1994). "Molecular cloning and characterization of a parathyroid hormone/parathyroid hormone-related peptide receptor: a member of an ancient family of G protein-coupled receptors." Curr Opin Nephrol Hypertens 3(4): 371-8.

Jüppner, H., A. B. Abou-Samra, M. Freeman, X. F. Kong, E. Schipani, J. Richards, L. F. Kolakowski, Jr., J. Hock, J. T. Potts, Jr., H. M. Kronenberg and et al. (1991). "A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide." Science 254(5034): 1024-6.

Jüppner, H., E. Brown and H. Kronenberg (1999). Parathyroid Hormone. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. M. Favus. Philadelphia, Lippincott, Williams &Wilkins: 80-87.

Jüppner, H., E. Schipani, M. Bastepe, D. E. Cole, M. L. Lawson, M. Mannstadt, G. N. Hendy, H. Plotkin, H. Koshiyama, T. Koh, J. D. Crawford, B. R. Olsen and M. Vikkula (1998). "The gene responsible for pseudohypoparathyroidism type Ib is paternally imprinted and maps in four unrelated kindreds to chromosome 20q13.3." Proc Natl Acad Sci U S A 95(20): 11798-803.

Jüppner, H., E. Schipani, F. R. Bringhurst, I. McClure, H. T. Keutmann, J. T. Potts, Jr., H. M. Kronenberg, A. B. Abou-Samra, G. V. Segre and T. J. Gardella (1994). "The extracellular amino-terminal region of the parathyroid hormone (PTH)/PTH-related peptide receptor determines the binding affinity for carboxyl-terminal fragments of PTH-(1-34)." Endocrinology 134(2): 879-84.

Kania, D. M., N. Binkley, M. Checovich, T. Havighurst, M. Schilling and W. B. Ershler (1995). "Elevated plasma levels of interleukin-6 in postmenopausal women do not correlate with bone density." J Am Geriatr Soc 43(3): 236-9.

Kanis, J. A. and S. Adami (1994). "Bone loss in the elderly." Osteoporos Int 4 Suppl 1: 59-65.

Kanis, J. A., L. J. r. Melton, C. Christiansen, C. C. Johnston and N. Khaltaev (1994). "The diagnosis of osteoporosis." J Bone Miner Res 9(8): 1137-41.

Kannus, P., M. Palvanen, J. Kaprio, J. Parkkari and M. Koskenvuo (1999). "Genetic factors and osteoporotic fractures in elderly people: prospective 25 year follow up of a nationwide cohort of elderly Finnish twins " Bmj 319(7221): 1334-7.

Karaplis, A. C., B. He, M. T. Nguyen, I. D. Young, D. Semeraro, H. Ozawa and N. Amizuka (1998). "Inactivating mutation in the human parathyroid hormone receptor type 1 gene in Blomstrand chondrodysplasia " Endocrinology 139(12): 5255-8.

Karaplis, A. C., A. Luz, J. Glowacki, R. T. Bronson, V. L. Tybulewicz, H. M. Kronenberg and R. C. Mulligan (1994). "Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene." Genes Dev 8(3): 277-89.

Karp, S. J., E. Schipani, B. St-Jacques, J. Hunzelman, H. Kronenberg and A. P. McMahon (2000). "Indian hedgehog coordinates endochondral bone growth and morphogenesis via parathyroid hormone related-protein-dependent and -independent pathways." Development 127(3): 543-8.

Karperien, M., H. J. van der Harten, R. van Schooten, H. Farih-Sips, N. S. den Hollander, S. L. Kneppers, P. Nijweide, S. E. Papapoulos and C. W. Lowik (1999). "A frame-shift mutation in the type I parathyroid hormone (PTH)/PTH-related peptide receptor causing Blomstrand lethal osteochondrodysplasia." J Clin Endocrinol Metab 84(10): 3713-20.

Kearsey, M. J. and H. S. Pooni (1996). The Genetical Analysis of Quantitative Traits. London, Chapman and Hall.

Keen, R. W., P. J. Major, J. S. Lanchbury and T. D. Spector (1995). "Vitamin-D-receptor-gene polymorphism and bone loss." Lancet 345(8955): 990.

Keen, R. W., H. Snieder, H. Molloy, J. Daniels, M. Chiano, F. Gibson, L. Fairbairn, P. Smith, A. J. MacGregor, D. Gewert and T. D. Spector (2001). "Evidence of association and linkage disequilibrium between a novel polymorphism in the transforming growth factor beta 1 gene and hip bone mineral density: a study of female twins." Rheumatology (Oxford) 40(1): 48-54.

Keen, R. W., K. L. Woodford-Richens, S. F. Grant, S. H. Ralston, J. S. Lanchbury and T. D. Spector (1999). "Association of polymorphism at the type I collagen (COL1A1) locus with reduced bone mineral density, increased fracture risk, and increased collagen turnover." Arthritis Rheum 42(2): 285-90.

Keen, R. W., K. L. Woodford-Richens, J. S. Lanchbury and T. D. Spector (1998). "Allelic variation at the interleukin-1 receptor antagonist gene is associated with early postmenopausal bone loss at the spine." Bone 23(4): 367-71.

Keene, D. R., L. Y. Sakai and R. E. Burgeson (1991). "Human bone contains type III collagen, type VI collagen, and fibrillin: type III collagen is present on specific fibers that may mediate attachment of tendons, ligaments, and periosteum to calcified bone cortex." J Histochem Cytochem 39(1): 59-69.

Kelly, P. J., J. L. Hopper, G. T. Macaskill, N. A. Pocock, P. N. Sambrook and J. A. Eisman (1991). "Genetic factors in bone turnover." J Clin Endocrinol Metab 72(4): 808-13.

Kelly, P. J., T. Nguyen, J. Hopper, N. Pocock, P. Sambrook and J. Eisman (1993). "Changes in axial bone density with age: a twin study." J Bone Miner Res 8(1): 11-7.

Kelly, T. (1990). "Bone mineral density reference databases for American men and women." J Bone Miner Res 5: S249.

Kemper, B. (1986). "Molecular biology of parathyroid hormone." CRC Crit Rev Biochem 19(4): 353-79.

Kerner, S., R. Scott and J. Wesley Pike (1989). "Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D3." Proc Natl Acad Sci USA 86: 4455-59.

Khosla, S., L. J. Melton, 3rd, E. J. Atkinson, W. M. O'Fallon, G. G. Klee and B. L. Riggs (1998). "Relationship of serum sex steroid levels and bone turnover markers with bone mineral density in men and women: a key role for bioavailable estrogen." J Clin Endocrinol Metab 83(7): 2266-74.

Khosla, S., J. M. Peterson, K. Egan, J. D. Jones and B. L. Riggs (1994). "Circulating cytokine levels in osteoporotic and normal women." J Clin Endocrinol Metab 79(3): 707-11.

Khoury, M., T. Beaty and B. Cohen (1993). Fundamentals of genetic epidemiology. Oxford, Oxford University Press.

Kiel, D. P., R. H. Myers, L. A. Cupples, X. F. Kong, X. H. Zhu, J. Ordovas, E. J. Schaefer, D. T. Felson, D. Rush, P. W. Wilson, J. A. Eisman and M. F. Holick (1997). "The BsmI vitamin D receptor restriction fragment length polymorphism (bb) influences the effect of calcium intake on bone mineral density." J Bone Miner Res 12(7): 1049-57.

Kikuchi, R., T. Uemura, I. Gorai, S. Ohno and H. Minaguchi (1999). "Early and late postmenopausal bone loss is associated with BsmI vitamin D receptor gene polymorphism in japanese women." Calcif Tissue Int 64(2): 102-6.

Kimble, R., S. Srivastava and R. Pacifici (1997). "Estrogen inhibites macrophage TNF gene expression by modulation binding of transcription factors to the AP-1 binding site." J Bone Miner Res 12(Suppl 1): S441.

Kimble, R. B., S. Srivastava, F. P. Ross, A. Matayoshi and R. Pacifici (1996). "Estrogen deficiency increases the ability of stromal cells to support murine osteoclastogenesis via an interleukin-1and tumor necrosis factor-mediated stimulation of macrophage colony-stimulating factor production." J Biol Chem 271(46): 28890-7.

Kinyamu, H. K., J. C. Gallagher, J. A. Knezetic, H. F. DeLuca, J. M. Prahl and S. J. Lanspa (1997). "Effect of vitamin D receptor genotypes on calcium absorption, duodenal vitamin D receptor concentration, and serum 1,25 dihydroxyvitamin D levels in normal women." Calcif Tissue Int 60(6): 491-5.

Kirkwood, B. (1988). The Essentials of Medical Statistics. Oxford, Blackwell Scientific Publications.

Kishimoto, T., S. Akira, M. Narazaki and T. Taga (1995). "Interleukin-6 family of cytokines and gp130." Blood 86(4): 1243-54.

Kitahama, S., M. A. Gibson, G. Hatzinikolas, S. Hay, J. L. Kuliwaba, A. Evdokiou, G. J. Atkins and D. M. Findlay (2000). "Expression of fibrillins and other microfibril-associated proteins in human bone and osteoblast-like cells." Bone 27(1): 61-7.

Klein, B., X. G. Zhang, M. Jourdan, J. M. Boiron, M. Portier, Z. Y. Lu, J. Wijdenes, J. Brochier and R. Bataille (1990). "Interleukin-6 is the central tumor growth factor in vitro and in vivo in multiple myeloma." Eur Cytokine Netw 1(4): 193-201.

Kobayashi, K., N. Takahashi, E. Jimi, N. Udagawa, M. Takami, S. Kotake, N. Nakagawa, M. Kinosaki, K. Yamaguchi, N. Shima, H. Yasuda, T. Morinaga, K. Higashio, T. J. Martin and T. Suda (2000). "Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction." J Exp Med 191(2): 275-86.

Kobayashi, S., S. Inoue, T. Hosoi, Y. Ouchi, M. Shiraki and H. Orimo (1996). "Association of bone mineral density with polymorphism of the estrogen receptor gene." J Bone Miner Res 11(3): 306-11.

Kobayashi, Y., F. Hashimoto, H. Miyamoto, K. Kanaoka, Y. Miyazaki-Kawashita, T. Nakashima, M. Shibata, K. Kobayashi, Y. Kato and H. Sakai (2000). "Force-induced osteoclast apoptosis in vivo is accompanied by elevation in transforming growth factor beta and osteoprotegerin expression " J Bone Miner Res 15(10): 1924-34.

Kodama, H., A. Yamasaki, M. Nose, S. Niida, Y. Ohgame, M. Abe, M. Kumegawa and T. Suda (1991). "Congenital osteoclast deficiency in osteopetrotic (op/op) mice is cured by injections of macrophage colony-stimulating factor." J Exp Med 173(1): 269-72.

Kohri, K., S. Nomura, Y. Kitamura, T. Nagata, K. Yoshioka, M. Iguchi, T. Yamate, T. Umekawa, Y. Suzuki, H. Sinohara and et al. (1993). "Structure and expression of the mRNA encoding urinary stone protein (osteopontin)." J Biol Chem 268(20): 15180-4.

Kohri, K., Y. Suzuki, K. Yoshida, K. Yamamoto, N. Amasaki, T. Yamate, T. Umekawa, M. Iguchi, H. Sinohara and T. Kurita (1992). "Molecular cloning and sequencing of cDNA encoding urinary stone protein, which is identical to osteopontin." Biochem Biophys Res Commun 184(2): 859-64.

Koller, D. L., M. J. Econs, P. A. Morin, J. C. Christian, S. L. Hui, P. Parry, M. E. Curran, L. A. Rodriguez, P. M. Conneally, G. Joslyn, M. Peacock, C. C. Johnston and T. Foroud (2000). "Genome screen for QTLs contributing to normal variation in bone mineral density and osteoporosis." J Clin Endocrinol Metab 85(9): 3116-20.

Koller, D. L., L. A. Rodriguez, J. C. Christian, C. W. Slemenda, M. J. Econs, S. L. Hui, P. Morin, P. M. Conneally, G. Joslyn, M. E. Curran, M. Peacock, C. C. Johnston and T. Foroud (1998). "Linkage of a QTL contributing to normal variation in bone mineral density to chromosome 11q12-13." J Bone Miner Res 13(12): 1903-8.

Kong, Y. Y., H. Yoshida, I. Sarosi, H. L. Tan, E. Timms, C. Capparelli, S. Morony, A. J. Oliveira-dos-Santos, G. Van, A. Itie, W. Khoo, A. Wakeham, C. R. Dunstan, D. L. Lacey, T. W. Mak, W. J. Boyle and J. M. Penninger (1999). "OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis." Nature 397(6717): 315-23.

Korach, K. S., J. F. Couse, S. W. Curtis, T. F. Washburn, J. Lindzey, K. S. Kimbro, E. M. Eddy, S. Migliaccio, S. M. Snedeker, D. B. Lubahn, D. W. Schomberg and E. P. Smith (1996). "Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes." Recent Prog Horm Res 51: 159-86; discussion 186-8.

Kosambi, D. (1944). "The estimation of map distances from recombination values." Annals of Eugenics 12: 172-75.

Krall, E. A. and B. Dawson-Hughes (1993). "Heritable and life-style determinants of bone mineral density." J Bone Miner Res 8(1): 1-9.

Krall, E. A., P. Parry, J. B. Lichter and B. Dawson-Hughes (1995). "Vitamin D receptor alleles and rates of bone loss: influences of years since menopause and calcium intake." J Bone Miner Res 10(6): 978-84.

Krege, J. H., J. B. Hodgin, J. F. Couse, E. Enmark, M. Warner, J. F. Mahler, M. Sar, K. S. Korach, J. A. Gustafsson and O. Smithies (1998). "Generation and reproductive phenotypes of mice lacking estrogen receptor beta." Proc Natl Acad Sci U S A 95(26): 15677-82.

Kruglyak, L. (1999). "Prospects for whole-genome linkage disequilibrium mapping of common disease genes." Nat Genet 22(2): 139-44.

Kruglyak, L., M. J. Daly, M. P. Reeve Daly and E. S. Lander (1996). "Parametric and nonparametric linkage analysis: a unified multipoint approach." Am J Hum Genet 58(6): 1347-63.

Kruglyak, L. and E. Lander (1995). "High-resolution genetic mapping of complex traits." Am J Hum Genet 56: 1212-1223.

Kruglyak, L. and E. S. Lander (1995). "Complete multipoint sib-pair analysis of qualitative and quantitative traits." Am J Hum Genet 57(2): 439-54.

Kruse, K. and C. Schutz (1993). "Calcium metabolism in the Jansen type of metaphyseal dysplasia." Eur J Pediatr 152(11): 912-5.

Kuivaniemi, H., G. Tromp and D. J. Prockop (1997). "Mutations in fibrillar collagens (types I, II, III, and XI), fibril-associated collagen (type IX), and network-forming collagen (type X) cause a spectrum of diseases of bone, cartilage, and blood vessels." Hum Mutat 9(4): 300-15.

Kuklin, A., K. Munson, D. Gjerde, R. Haefele and P. Taylor (1997). "Detection of single-nucleotide polymorphisms with the WAVE DNA fragment analysis system." Genet Test 1(3): 201-6.

Kumegawa, M., M. Hiramatsu, K. Hatakeyama, T. Yajima, H. Kodama, T. Osaki and K. Kurisu (1983). "Effects of epidermal growth factor on osteoblastic cells in vitro." Calcif Tissue Int 35(4-5): 542-8.

Kurland, E., C. Rosen and F. e. a. Cosman (1997). "Insulin-like growth factor-1 in men with idiopathic osteoporosis." J Clin Endocrinol Metab 82(9): 2799-2805.

Lacey, D. L., E. Timms, H. L. Tan, M. J. Kelley, C. R. Dunstan, T. Burgess, R. Elliott, A. Colombero, G. Elliott, S. Scully, H. Hsu, J. Sullivan, N. Hawkins, E. Davy, C. Capparelli, A. Eli, Y. X. Qian, S. Kaufman, I. Sarosi, V. Shalhoub, G. Senaldi, J. Guo, J. Delaney and W. J. Boyle (1998). "Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation." Cell 93(2): 165-76.

Lander, E. and L. Kruglyak (1995). "Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results." Nat Genet 11(3): 241-7.

Landis, W. (1995). "The strength of a calcified tissue depends in part on the molecular strucrture and organsization of its constituent mineral crystals in their organic matrix." Bone 16: 533-44.

Langdahl, B., M. Carstens, L. Stenkjaer and E. Eriksen (2000). "Polymorphisms in the Osteoprotegerin Gene and Osteoporotic Fractures and Bone Mass." J Bone Miner Res 15(Suppl 1): F159.

Langdahl, B. L., C. H. Gravholt, K. Brixen and E. F. Eriksen (2000). "Polymorphisms in the vitamin D receptor gene and bone mass, bone turnover and osteoporotic fractures " Eur J Clin Invest 30(7): 608-17.

Langdahl, B. L., J. Y. Knudsen, H. K. Jensen, N. Gregersen and E. F. Eriksen (1997). "A sequence variation: 713-8delC in the transforming growth factor-beta 1 gene has higher prevalence in osteoporotic women than in normal women and is associated with very low bone mass in osteoporotic women and increased bone turnover in both osteoporotic and normal women." Bone 20(3): 289-94.

Langdahl, B. L., E. Lokke, M. Carstens, L. L. Stenkjaer and E. F. Eriksen (2000). "Osteoporotic fractures are associated with an 86-base pair repeat polymorphism in the interleukin-1--receptor antagonist gene but not with polymorphisms in the interleukin-1beta gene." J Bone Miner Res 15(3): 402-14.

Langdahl, B. L., E. Lokke, M. Carstens, L. L. Stenkjaer and E. F. Eriksen (2000). "A TA repeat polymorphism in the estrogen receptor gene is associated with osteoporotic fractures but polymorphisms in the first exon and intron are not " J Bone Miner Res 15(11): 2222-30.

Langdahl, B. L., S. H. Ralston, S. F. Grant and E. F. Eriksen (1998). "An Sp1 binding site polymorphism in the COLIA1 gene predicts osteoporotic fractures in both men and women." J Bone Miner Res 13(9): 1384-9.

Langub, M. C., M. C. Monier-Faugere, Q. Qi, Z. Geng, N. J. Koszewski and H. H. Malluche (2001). "Parathyroid hormone/parathyroid hormone-related peptide type 1 receptor in human bone." J Bone Miner Res 16(3): 448-56.

Lanske, B., M. Amling, L. Neff, J. Guiducci, R. Baron and H. M. Kronenberg (1999). "Ablation of the PTHrP gene or the PTH/PTHrP receptor gene leads to distinct abnormalities in bone development." J Clin Invest 104(4): 399-407.

Lathrop, G. M. (1983). "Estimating genotype relative risks." Tissue Antigens 22(2): 160-6.

Lathrop, G. M. and J. M. Lalouel (1984). "Easy calculations of lod scores and genetic risks on small computers." Am J Hum Genet 36: 460-465.

Lawrence, J. S., C. L. Martins and G. L. Drake (1987). "A family survey of lupus erythematosus. 1. Heritability." J Rheumatol 14(5): 913-21.

Leung, D. and R. Geha (1988). "Clinical and immunologic aspects of the hyperimmunoglobulin E syndrome." Hemat. Oncol. Clin. North Am. 2: 81-100.

Lewis, D. B., H. D. Liggitt, E. L. Effmann, S. T. Motley, S. L. Teitelbaum, K. J. Jepsen, S. A. Goldstein, J. Bonadio, J. Carpenter and R. M. Perlmutter (1993). "Osteoporosis induced in mice by overproduction of interleukin 4." Proc Natl Acad Sci U S A 90(24): 11618-22.

Li, Y. C., M. Amling, A. E. Pirro, M. Priemel, J. Meuse, R. Baron, G. Delling and M. B. Demay (1998). "Normalization of mineral ion homeostasis by dietary means prevents hyperparathyroidism, rickets, and osteomalacia, but not alopecia in vitamin D receptor-ablated mice." Endocrinology 139(10): 4391-6.

Li, Y. C., A. E. Pirro, M. Amling, G. Delling, R. Baron, R. Bronson and M. B. Demay (1997). "Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia." Proc Natl Acad Sci U S A 94(18): 9831-5.

Lian, J., G. Stein, E. Canalis, P. Robey and A. Boskey (1999). Bone formation: osteoblast lineage cells, growth factors, matrix proteins and the mineralization process. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. M. Favus. Philadelphia, Lippincott Williams and Wilkins: 14-27.

Liaw, L., D. E. Birk, C. B. Ballas, J. S. Whitsitt, J. M. Davidson and B. L. Hogan (1998). "Altered wound healing in mice lacking a functional osteopontin gene (spp1)." J Clin Invest 101(7): 1468-78.

Liberman, U. and S. Marx (1999). Vitamin D-dependent rickets. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. M. Favus. Philadelphia, Lippincott, Williams and Wilkins: 323-328.

Liden, M., B. Wilen, S. Ljunghall and H. Melhus (1998). "Polymorphism at the Sp 1 binding site in the collagen type I alpha 1 gene does not predict bone mineral density in postmenopausal women in sweden." Calcif Tissue Int 63(4): 293-5.

Lin, S. C., T. Yamate, Y. Taguchi, V. Z. Borba, G. Girasole, C. A. O'Brien, T. Bellido, E. Abe and S. C. Manolagas (1997). "Regulation of the gp80 and gp130 subunits of the IL-6 receptor by sex steroids in the murine bone marrow." J Clin Invest 100(8): 1980-90.

Liu, W., D. I. Smith, K. J. Rechtzigel, S. N. Thibodeau and C. D. James (1998). "Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations." Nucleic Acids Res 26(6): 1396-400.

Lofman, O., L. Larsson and G. Toss (2000). "Bone mineral density in diagnosis of osteoporosis: reference population, definition of peak bone mass, and measured site determine prevalence." J Clin Densitom 3(2): 177-86.

Looker, A. C., E. S. Orwoll, C. C. Johnston, R. L. Lindsay, H. W. Wahner, W. L. Dunn, M. S. Calvo, T. B. Harris and S. P. Heyse (1997). "Prevalence of low femoral bone density in older U.S. adults from NHANES III." J Bone Miner Res 12(11): 1761-8.

Looker, A. C., H. W. Wahner, W. L. Dunn, M. S. Calvo, T. B. Harris, S. P. Heyse, C. C. Johnston and R. Lindsay (1998). "Updated data on proximal femur bone mineral levels of US adults." Osteoporos Int 8(5): 468-89.

Looker, A. C., H. W. Wahner, W. L. Dunn, M. S. Calvo, T. B. Harris, S. P. Heyse, C. C. Johnston and R. L. Lindsay (1995). "Proximal femur bone mineral levels of US adults." Osteoporos Int 5(5): 389-409.

Lorentzon, M., R. Lorentzon and P. Nordstrom (2000). "Interleukin-6 gene polymorphism is related to bone mineral density during and after puberty in healthy white males: a cross-sectional and longitudinal study " J Bone Miner Res 15(10): 1944-9.

Lorenzo, J. A., A. Naprta, Y. Rao, C. Alander, M. Glaccum, M. Widmer, G. Gronowicz, J. Kalinowski and C. C. Pilbeam (1998). "Mice lacking the type I interleukin-1 receptor do not lose bone mass after ovariectomy " Endocrinology 139(6): 3022-5.

Lubberts, E., L. A. Joosten, M. Chabaud, L. van Den Bersselaar, B. Oppers, C. J. Coenen-De Roo, C. D. Richards, P. Miossec and W. B. van Den Berg (2000). "IL-4 gene therapy for collagen arthritis suppresses synovial IL-17 and osteoprotegerin ligand and prevents bone erosion." J Clin Invest 105(12): 1697-710.

MacGregor, A., H. Snieder and T. D. Spector (2000). "Genetic factors and osteoporotic fractures in elderly people. Twin data support genetic contribution to risk of fracture [letter; comment]." Bmj 320(7250): 1669-70; discussion 1670-1.

MacGregor, A. J., L. Antoniades, M. Matson, T. Andrew and T. D. Spector (2000). "The genetic contribution to radiographic hip osteoarthritis in women: results of a classic twin study." Arthritis Rheum 43(11): 2410-6.

MacGregor, A. J., H. Snieder, A. S. Rigby, M. Koskenvuo, J. Kaprio, K. Aho and A. J. Silman (2000). "Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins." Arthritis Rheum 43(1): 30-7.

Manen, D., G. Palmer, J. P. Bonjour and R. Rizzoli (1998). "Sequence and activity of parathyroid hormone/parathyroid hormone- related protein receptor promoter region in human osteoblast-like cells." Gene 218(1-2): 49-56.

Mannstadt, M., H. Jüppner and T. J. Gardella (1999). "Receptors for PTH and PTHrP: their biological importance and functional properties." Am J Physiol 277(5 Pt 2): F665-75.

Manolagas, S. and R. Jilka (1995). "Bone marrow, cytokines and bone remodelling: emerging insights into the pathophysiology of osteoporosis." New Engl J Med 332(5): 305-317.

Manolagas, S. C. (2000). "Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis." Endocr Rev 21(2): 115-37.

Marcus, R., D. Leary, D. L. Schneider, E. Shane, M. Favus and C. A. Quigley (2000). "The contribution of testosterone to skeletal development and maintenance: lessons from the androgen insensitivity syndrome." J Clin Endocrinol Metab 85(3): 1032-7.

Maricic, M. and Z. Chen (2000). Bone Densitometry. Osteoporosis and Metabolic Bone Disease. C. Rosen. Philadelphia, W.B. Saunders. 20: 469-488.

Marlow, A. (2001). Non-Parametric Linkage Analysis II. Variance Components. Quantitative Trait Loci: Methods and Protocols. N. a. C. Camp, A, Humana Press Inc.: in press.

Marshall, D., O. Johnell and H. Wedel (1996). "Meta-analysis of how well measures of bone mineral density predict occurrence of osteoporotic fractures " Bmj 312(7041): 1254-9.

Martin, E. R., N. L. Kaplan and B. S. Weir (1997). "Tests for linkage and association in nuclear families." Am J Hum Genet 61(2): 439-48.

Masi, L., L. Becherini, E. Colli, L. Gennari, R. Mansani, A. Falchetti, A. M. Becorpi, C. Cepollaro, S. Gonnelli, A. Tanini and M. L. Brandi (1998). "Polymorphisms of the calcitonin receptor gene are associated with bone mineral density in postmenopausal Italian women." Biochem Biophys Res Commun 248(1): 190-5.

McCuaig, K. A., H. S. Lee, J. C. Clarke, H. Assar, J. Horsford and J. H. White (1995). "Parathyroid hormone/parathyroid hormone related peptide receptor gene transcripts are expressed from tissue-specific and ubiquitous promoters [published erratum appears in Nucleic Acids Res 1995 Aug 11;23(15):3082]." Nucleic Acids Res 23(11): 1948-55.

McGuigan, F. E., D. M. Reid and S. H. Ralston (2000). "Susceptibility to osteoporotic fracture is determined by allelic variation at the Sp1 site, rather than other polymorphic sites at the COL1A1 locus." Osteoporos Int 11(4): 338-43.

McKay, H. A., D. A. Bailey, A. A. Wilkinson and C. S. Houston (1994). "Familial comparison of bone mineral density at the proximal femur and lumbar spine." Bone Miner 24(2): 95-107.

McKinley, D., Q. Wu, T. Yang-Feng and Y. C. Yang (1992). "Genomic sequence and chromosomal location of human interleukin-11 gene (IL11)." Genomics 13(3): 814-9.

Melton, L. (1988). Epidemiology of fractures. Osteoporosis: Etiology, Diagnosis and Management. B. Riggs and L. Melton. New York, Raven Press: 133-54.

Melton, L. J., 3rd, E. J. Atkinson, M. K. O'Connor, W. M. O'Fallon and B. L. Riggs (1998). "Bone density and fracture risk in men." J Bone Miner Res 13(12): 1915-23.

Melton, L. J., 3rd, D. M. Ilstrup, R. D. Beckenbaugh, B. L. Riggs (1982). "Hip fracture recurrence. A population-based study." Clin Orthop 167: 131-8.

Melton, L. J., 3rd, S. Khosla, E. J. Atkinson, W. M. O'Fallon and B. L. Riggs (1997). "Relationship of bone turnover to bone density and fractures." J Bone Miner Res 12(7): 1083-91.

Melton, L. J. d., E. A. Chrischilles, C. Cooper, A. W. Lane and B. L. Riggs (1992). "Perspective. How many women have osteoporosis?" J Bone Miner Res 7(9): 1005-10.

Min, H., S. Morony, I. Sarosi, C. R. Dunstan, C. Capparelli, S. Scully, G. Van, S. Kaufman, P. J. Kostenuik, D. L. Lacey, W. J. Boyle and W. S. Simonet (2000). "Osteoprotegerin Reverses Osteoporosis by Inhibiting Endosteal Osteoclasts and Prevents Vascular Calcification by Blocking a Process Resembling Osteoclastogenesis." J Exp Med 192(4): 463-474.

Minagawa, M., M. Y. Kwan, J. D. Bettoun, F. W. Mansour, J. Dassa, G. N. Hendy, D. Goltzman and J. H. White (2000). "Dissection of differentially regulated (G+C)-rich promoters of the human parathyroid hormone (PTH)/PTH-related peptide receptor gene." Endocrinology 141(7): 2410-21.

Mizuno, A., N. Amizuka, K. Irie, A. Murakami, N. Fujise, T. Kanno, Y. Sato, N. Nakagawa, H. Yasuda, S. Mochizuki, T. Gomibuchi, K. Yano, N. Shima, N. Washida, E. Tsuda, T. Morinaga, K. Higashio and H. Ozawa (1998). "Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin." Biochem Biophys Res Commun 247(3): 610-5.

Mizunuma, H., T. Hosoi, H. Okano, M. Soda, T. Tokizawa, I. Kagami, S. Miyamoto, Y. Ibuki, S. Inoue, M. Shiraki and Y. Ouchi (1997). "Estrogen receptor gene polymorphism and bone mineral density at the lumbar spine of pre- and postmenopausal women." Bone 21(5): 379-83.

Moffatt, M. F., J. A. Traherne, G. R. Abecasis and W. O. Cookson (2000). "Single nucleotide polymorphism and linkage disequilibrium within the TCR alpha/delta locus." Hum Mol Genet 9(7): 1011-9.

Morinaga, T., N. Nakagawa, H. Yasuda, E. Tsuda and K. Higashio (1998). "Cloning and characterization of the gene encoding human osteoprotegerin/osteoclastogenesis-inhibitory factor." Eur J Biochem 254(3): 685-91.

Morrison, N. A., J. Cheng Qi, A. Tokita, P. J. Kelly, L. Crofts, T. V. Nguyen, P. N. Sambrook and J. A. Eisman (1994). "Prediction of bone mineral density from vitamin D receptor alleles." Nature 367: 284-287.

Morrison, N. A., J. C. Qi, A. Tokita, P. J. Kelly, L. Croft, T. V. Nguyen, P. N. Sambrook and J. A. Eisman (1997). "Prediction of bone density by vitamin D receptor alleles (correction)." Nature 387: 106.

Morrison, N. A., R. Yeoman, P. J. Kelly and J. A. Eisman (1992). "Contribution of trans-acting factor alleles to normal physiological variability: vitamin D receptor gene polymorphism and circulating osteocalcin." Proc Natl Acad Sci U S A 89(15): 6665-9.

Morton, N. and A. Collins (1998). "Tests and estimates of allelic association in complex inheritance." Proc. Natl. Acad. Sci. USA 95: 11389-11393.

Mullersman, J., J. Whields and B. Saha (1992). "Characterization of two novel polymorphism at the human parathyroid hormone gene locus." Hum. Genet. 88: 589-592.

Mundy, G. (1999). Bone remodeling. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. M. Favus. Philadelphia, Lippincott Williams &Wilkins: 30-38.

Murray, R., S. Grant, D. Reid and S. Ralston (1996). "Polymorphisms of the interleukin-6 gene are associated with bone mineral density." Osteoporosis Int 6(Suppl1): 89.

Myers, R. H., E. J. Schaefer, P. W. Wilson, R. D'Agostino, J. M. Ordovas, A. Espino, R. Au, R. F. White, J. E. Knoefel, J. L. Cobb, K. A. McNulty, A. Beiser and P. A. Wolf (1996). "Apolipoprotein E epsilon4 association with dementia in a population- based study: The Framingham study." Neurology 46(3): 673-7.

Nakashima, T., Y. Kobayashi, S. Yamasaki, A. Kawakami, K. Eguchi, H. Sasaki and H. Sakai (2000). "Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines." Biochem Biophys Res Commun 275(3): 768-75.

Neer, R. M., C. D. Arnaud, J. R. Zanchetta, R. Prince, G. A. Gaich, J. Y. Reginster, A. B. Hodsman, E. F. Eriksen, S. Ish-Shalom, H. K. Genant, O. Wang, B. H. Mitlak (2001). "Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis." N Engl J Med 344(19):1434-41.

Neugebauer, W., W. K. Surewicz, H. L. Gordon, R. L. Somorjai, W. Sung and G. E. Willick (1992). "Structural elements of human parathyroid hormone and their possible relation to biological activities." Biochemistry 31(7): 2056-63.

Nguyen, T. V., J. A. Eisman, P. J. Kelly and P. N. Sambrook (1996). "Risk factors for osteoporotic fractures in elderly men." Am J Epidemiol 144(3): 255-63.

Nguyen, T. V., G. M. Howard, P. J. Kelly and J. A. Eisman (1998). "Bone mass, lean mass, and fat mass: same genes or same environments?" Am J Epidemiol 147(1): 3-16.

Nicholson, G. C., J. M. Moseley, P. M. Sexton, F. A. Mendelsohn and T. J. Martin (1986). "Abundant calcitonin receptors in isolated rat osteoclasts. Biochemical and autoradiographic characterization." J Clin Invest 78(2): 355-60.

Nishida, S., A. Yamaguchi, T. Tanizawa, N. Endo, T. Mashiba, Y. Uchiyama, T. Suda, S. Yoshiki and H. E. Takahashi (1994). "Increased bone formation by intermittent parathyroid hormone administration is due to the stimulation of proliferation and differentiation of osteoprogenitor cells in bone marrow." Bone 15(6): 717-23.

Niu, T., C. Chen, H. Cordell, J. Yang, B. Wang, Z. Wang, Z. Fang, N. J. Schork, C. J. Rosen and X. Xu (1999). "A genome-wide scan for loci linked to forearm bone mineral density." Hum Genet 104(3): 226-33.

Noble, B., J. Routledge, H. Stevens, I. Hughes and W. Jacobson (1999). "Androgen receptors in bone-forming tissue." Horm Res 51(1): 31-6.

Nogues, X., N. Garcia-Giralt, A. Bay Jensen, A. Enjuanes, S. Quintana, L. Mellibovsky, J. Puig, S. Balcells, D. Grinberg and A. Diez (2000). "Linkage Disequili brium betwween COL1A1 Sp1and a new 1 bp deletion polymorphism in the upstream regulatory region of the gene in Spanish perimenopausal women." J Bone Miner Res 15(Suppl 1): S490.

Nordstrum, P. and R. Lorentzon (1999). "Influence of Heredity and Environment on Bone Density in Adolescent Boys: A Parent-Offspring Study." Osteoporosis Int 10: 271-277.

Nussenzveig, D. R., S. Mathew and M. C. Gershengorn (1995). "Alternative splicing of a 48-nucleotide exon generates two isoforms of the human calcitonin receptor." Endocrinology 136(5): 2047-51.

Nyholt, D. (2000). "All LODs are not created equal." Am J Hum Genet 67: 282-288.

O'Brien, C., N. Farrar and S. Manolagas (1998). "Identification of an OSF-2 binding site in the murine RANKL/OPGL gene promoter: a potential link between osteoblastogenesis and osteoclastogenesis." Bone 23(Suppl 1): S149.

O'Connell, J. R. and D. E. Weeks (1997). "PedCheck: A program for identifying marker typing incompatibilities in linkage analysis." Am J Hum Genet 61: A288.

O'Donovan, M. C., P. J. Oefner, S. C. Roberts, J. Austin, B. Hoogendoorn, C. Guy, G. Speight, M. Upadhyaya, S. S. Sommer and P. McGuffin (1998). "Blind analysis of denaturing high-performance liquid chromatography as a tool for mutation detection." Genomics 52(1): 44-9.

O'Neill, T. W., D. Felsenberg, J. Varlow, C. Cooper, J. A. Kanis and A. J. Silman (1996). "The prevalence of vertebral deformity in european men and women: the European Vertebral Osteoporosis Study." J Bone Miner Res 11(7): 1010-8.

Ongphiphadhanakul, B., R. Rajatanavin, S. Chanprasertyothin, N. Piaseu and L. Chailurkit (1998). "Serum oestradiol and oestrogen-receptor gene polymorphism are associated with bone mineral density independently of serum testosterone in normal males." Clin Endocrinol (Oxf) 49(6): 803-9.

Ongphiphadhanakul, B., R. Rajatanavin, S. Chanprasertyothin, N. Piaseu, L. Chailurkit, R. Sirisriro and S. Komindr (1998). "Estrogen receptor gene polymorphism is associated with bone mineral density in premenopausal women but not in postmenopausal women." J Endocrinol Invest 21(8): 487-93.

Orwoll, E. and R. Klein (1995). "Osteoporosis in Men." Endocrine Rev 16(1): 87-116.

Orwoll, E. S. (1998). "Osteoporosis in men." Endocrinol Metab Clin North Am 27(2): 349-67.

Ota, N., S. Hunt, T. Nakajima, T. Suzuki, T. Hosoi, H. Orimo, Y. Shirai and M. Emi (2000). "Linkage of human tumor necrosis factor-alpha to human osteoporosis by sib-pair analysis." Genes and Immunity 1: 260-264.

Ota, N., S. C. Hunt, T. Nakajima, T. Suzuki, T. Hosoi, H. Orimo, Y. Shirai and M. Emi (1999). "Linkage of interleukin 6 locus to human osteopenia by sibling pair analysis." Hum Genet 105(3): 253-7.

Otto, F., A. Thornell, T. Crompton and e. al (1997). "Cbfa-1, a candidate gene for cleidocranial dysplasia syndrome, is essential fro osteoblast differentiation and bone development." Cell 89: 765-771.

Pacifici, R. (1996). "Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis." J Bone Miner Res 11(8): 1043-51.

Palmer, L. J., P. R. Burton, A. L. James, A. W. Musk and W. O. Cookson (2000). "Familial aggregation and heritability of asthma-associated quantitative traits in a population-based sample of nuclear families." Eur J Hum Genet 8(11): 853-60.

Parfitt, A. M., E. Schipani, D. S. Rao, W. Kupin, Z. H. Han and H. Jüppner (1996). "Hypercalcemia due to constitutive activity of the parathyroid hormone (PTH)/PTH-related peptide receptor: comparison with primary hyperparathyroidism." J Clin Endocrinol Metab 81(10): 3584-8.

Parkinson, D. B. and R. V. Thakker (1992). "A donor splice site mutation in the parathyroid hormone gene is associated with autosomal recessive hypoparathyroidism." Nat Genet 1(2): 149-52.

Parsian, A., R. D. Todd, E. J. Devor, K. L. O'Malley, B. K. Suarez, T. Reich and C. R. Cloninger (1991). "Alcoholism and alleles of the human D2 dopamine receptor locus. Studies of association and linkage." Arch Gen Psychiatry 48(7): 655-63.

Passeri, G., G. Girasole, S. C. Manolagas and R. L. Jilka (1994). "Endogenous production of tumor necrosis factor by primary cultures of murine calvarial cells: influence on IL-6 production and osteoclast development." Bone Miner 24(2): 109-26.

Patel, M. S., D. E. Cole, J. D. Smith, G. A. Hawker, B. Wong, H. Trang, R. Vieth, P. Meltzer and L. A. Rubin (2000). "Alleles of the estrogen receptor alpha-gene and an estrogen receptor cotranscriptional activator gene, amplified in breast cancer-1 (AIB1), are associated with quantitative calcaneal ultrasound " J Bone Miner Res 15(11): 2231-9.

Peacock, M. (1995). "Vitamin D receptor gene alleles and osteoporosis: a contrasting view [editorial]." J Bone Miner Res 10(9): 1294-7.

Pearce, S. H. and R. V. Thakker (1997). "The calcium-sensing receptor: insights into extracellular calcium homeostasis in health and disease." J Endocrinol 154(3): 371-8.

Pedeutour, F., S. Merscher and E. e. a. Durieux (1994). "Mapping of the 12q12-q22 region with respect to tumor translocation breakpoints." Genomics 22: 512-8.

Pellegrini, M., M. Royo, M. Rosenblatt, M. Chorev and D. F. Mierke (1998). "Addressing the tertiary structure of human parathyroid hormone-(1-34)." J Biol Chem 273(17): 10420-7.

Penrose, L. (1935). "The general purpose sib-pair linkage test." Ann Eugenics 6: 133-138.

Pericak-Vance, M. A., J. L. Bebout, P. C. Gaskell, Jr., L. H. Yamaoka, W. Y. Hung, M. J. Alberts, A. P. Walker, R. J. Bartlett, C. A. Haynes, K. A. Welsh and et al. (1991). "Linkage studies in familial Alzheimer disease: evidence for chromosome 19 linkage." Am J Hum Genet 48(6): 1034-50.

Pfeilschifter, J., C. Chenu, A. Bird, G. R. Mundy and G. D. Roodman (1989). "Interleukin-1 and tumor necrosis factor stimulate the formation of human osteoclastlike cells in vitro." J Bone Miner Res 4(1): 113-8.

Pfeilschifter, J., I. Diel, B. Scheppach, A. Bretz, R. Krempien, J. Erdmann, G. Schmid, N. Reske, H. Bismar, T. Seck, B. Krempien and R. Ziegler (1998). "Concentration of transforming growth factor beta in human bone tissue: relationship to age, menopause, bone turnover, and bone volume." J Bone Miner Res 13(4): 716-30.

Pociot, F., J. Molvig, L. Wogensen, H. Worsaae and J. Nerup (1992). "A TaqI polymorphism in the human interleukin-1 beta (IL-1 beta) gene correlates with IL-1 beta secretion in vitro." Eur J Clin Invest 22(6): 396-402.

Pocock, N., J. Eisman, J. Hopper, M. Yeates, P. Sambrook and S. Eberl (1987). "Genetic determinants of bone mass in adults. A twin study." J Clin Invest 80(3): 706-710.

Pocock, N. A., P. N. Sambrook, T. Nguyen, P. Kelly, J. Freund and J. A. Eisman (1992). "Assessment of spinal and femoral bone density by dual X-ray absorptiometry: comparison of lunar and hologic instruments." J Bone Miner Res 7(9): 1081-4.

Poli, V., R. Balena, E. Fattori, A. Markatos, M. Yamamoto, H. Tanaka, G. Ciliberto, G. A. Rodan and F. Costantini (1994). "Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion." Embo J 13(5): 1189-96.

Pollak, M. R., E. M. Brown, Y. H. Chou, S. C. Hebert, S. J. Marx, B. Steinmann, T. Levi, C. E. Seidman and J. G. Seidman (1993). "Mutations in the human Ca(2+)-sensing receptor gene cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism " Cell 75(7): 1297-303.

Pollak, M. R., E. M. Brown, H. L. Estep, P. N. McLaine, O. Kifor, J. Park, S. C. Hebert, C. E. Seidman and J. G. Seidman (1994). "Autosomal dominant hypocalcaemia caused by a Ca(2+)-sensing receptor gene mutation." Nat Genet 8(3): 303-7.

Pollak, M. R., Y. H. Chou, S. J. Marx, B. Steinmann, D. E. Cole, M. L. Brandi, S. E. Papapoulos, F. H. Menko, G. N. Hendy, E. M. Brown and et al. (1994). "Familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. Effects of mutant gene dosage on phenotype." J Clin Invest 93(3): 1108-12.

Ponglikitmongkol, M., S. Green and P. Chambon (1988). "Genomic organization of the human oestrogen receptor gene." Embo J 7(11): 3385-8.

Prockop, D. and K. Kivirikko (1995). "Collagens: molecular biology, diseases and potentials for therapy." Annu. Rev. Biochem. 64: 403-34.

Ragoussis, J., K. Bloemer, E. H. Weiss and A. Ziegler (1988). "Localization of the genes for tumor necrosis factor and lymphotoxin between the HLA class I and III regions by field inversion gel electrophoresis." Immunogenetics 27(1): 66-9.

Reed, P. W., J. L. Davies, J. B. Copeman, S. T. Bennett, S. M. Palmer, L. E. Pritchard, S. C. Gough, Y. Kawaguchi, H. J. Cordell, K. M. Balfour and et al. (1994). "Chromosome-specific microsatellite sets for fluorescence-based, semi-automated genome mapping." Nat Genet 7(3): 390-5.

Reginster, J. Y., R. Deroisy, M. P. Lecart, N. Sarlet, B. Zegels, I. Jupsin, M. de Longueville and P. Franchimont (1995). "A double-blind, placebo-controlled, dose-finding trial of intermittent nasal salmon calcitonin for prevention of postmenopausal lumbar spine bone loss." Am J Med 98(5): 452-8.

Reinholt, F. P., K. Hultenby, A. Oldberg and D. Heinegard (1990). "Osteopontin--a possible anchor of osteoclasts to bone." Proc Natl Acad Sci U S A 87(12): 4473-5.

Riancho, J. A., M. T. Zarrabeitia, G. R. Mundy, T. Yoneda and J. Gonzalez-Macias (1993). "Effects of interleukin-4 on the formation of macrophages and osteoclast-like cells." J Bone Miner Res 8(11): 1337-44.

Rigby, A. S., A. J. MacGregor and G. Thomson (1998). "HLA haplotype sharing in rheumatoid arthritis sibships: risk estimates subdivided by proband genotype." Genet Epidemiol 15(4): 403-18.

Riggs, B. and T. A. S. o. B. a. M. R. P. s. c. o. nomenclature (2000). "Proposed Standard Nomenclature for new Tumor Necrosis Factor Family Members Involved in the Regulation of Bone Resorption." J Bone Miner Res 15(12): 2293-96.

Riis, B. J., M. A. Hansen, A. M. Jensen, K. Overgaard and C. Christiansen (1996). "Low bone mass and fast rate of bone loss at menopause: equal risk factors for future fracture: a 15-year follow-up study." Bone 19(1): 9-12.

Risch, N. (1990). "Linkage strategies for genetically complex traits. II. The power of affected relative pairs " Am J Hum Genet 46(2): 229-41.

Risch, N. (1990). "Linkage strategies for genetically complex traits. III. The effect of marker polymorphism on analysis of affected relative pairs [published erratum appears in Am J Hum Genet 1992 Sep;51(3):673-5] " Am J Hum Genet 46(2): 242-53.

Risch, N. (2000). "Searching for genetic determinants in the new millennium." Nature 454: 847-856.

Risch, N. and K. Merikangas (1996). "The future of genetic studies of complex human diseases." Science 273: 1516-7.

Risch, N. and H. Zhang (1995). "Extreme discordant sib pairs for mapping quantitative trait loci in humans." Science 268: 1584-1589.

Ritvaniemi, P., J. Korkko, J. Bonaventure, M. Vikkula, J. Hyland, P. Paassilta, I. Kaitila, H. Kaariainen, B. P. Sokolov, M. Hakala and et al. (1995). "Identification of COL2A1 gene mutations in patients with chondrodysplasias and familial osteoarthritis." Arthritis Rheum 38(7): 999-1004.

Roberts, S. B., C. J. MacLean, M. C. Neale, L. J. Eaves and K. S. Kendler (1999). "Replication of linkage studies of complex traits: an examination of variation in location estimates." Am J Hum Genet 65(3): 876-84.

Rogaev, E. I., R. Sherrington, E. A. Rogaeva, G. Levesque, M. Ikeda, Y. Liang, H. Chi, C. Lin, K. Holman, T. Tsuda and et al. (1995). "Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene." Nature 376(6543): 775-8.

Romas, E., N. Udagawa, H. Zhou, T. Tamura, M. Saito, T. Taga, D. J. Hilton, T. Suda, K. W. Ng and T. J. Martin (1996). "The role of gp130-mediated signals in osteoclast development: regulation of interleukin 11 production by osteoblasts and distribution of its receptor in bone marrow cultures." J Exp Med 183(6): 2581-91.

Rosen, C. J., E. S. Kurland, D. Vereault, R. A. Adler, P. J. Rackoff, W. Y. Craig, S. Witte, J. Rogers and J. P. Bilezikian (1998). "Association between serum insulin growth factor-I (IGF-I) and a simple sequence repeat in IGF-I gene: implications for genetic studies of bone mineral density." J Clin Endocrinol Metab 83(7): 2286-90.

Ryan, P., T. Spector, G. Blake, D. Doyle and I. Fogelman (1993). "A comparison of reference bone mineral density measurements derived from two sources: referred and population based." Br J Radiol 66(792): 1138-41.

Ryan, P. J., T. P. Spector, G. M. Blake, D. V. Doyle and I. Fogelman (1993). "A comparison of reference bone mineral density measurements derived from two sources: referred and population based." Br J Radiol 66(792): 1138-41.

Sainz, J., J. M. Van Tornout, J. Sayre, F. Kaufman and V. Gilsanz (1999). "Association of collagen type 1 alpha1 gene polymorphism with bone density in early childhood." J Clin Endocrinol Metab 84(3): 853-5.

Salamone, L., J. Cauley, J. Zmuda, A. Pasagian-Macaulay, R. Epstein, R. Ferrell, D. Black and L. Kuller (2000). "Apolipoprotein E Gene Polymorphism and Bone Loss: Estrogen Status Modifies the Influence of Apolipoprotein E on Bone Loss." J Bone Miner Res 15(2): 308-314.

Salmen, T., A. M. Heikkinen, A. Mahonen, H. Kroger, M. Komulainen, S. Saarikoski, R. Honkanen and P. H. Maenpaa (2000). "Early postmenopausal bone loss is associated with PvuII estrogen receptor gene polymorphism in Finnish women: effect of hormone replacement therapy." J Bone Miner Res 15(2): 315-21.

Sambrook, J., E. Fritsch and T. Maniatis (1989). Molecular Cloning: A Laboratory Manual. New York, Cold Spring Harbor Laboratory Press.

Santtila, S., K. Savinainen and M. Hurme (1998). "Presence of the IL-1RA allele 2 (IL1RN*2) is associated with enhanced IL-1beta production in vitro." Scand J Immunol 47(3): 195-8.

Saunders, A. M., W. J. Strittmatter, D. Schmechel, P. H. George-Hyslop, M. A. Pericak-Vance, S. H. Joo, B. L. Rosi, J. F. Gusella, D. R. Crapper-MacLachlan, M. J. Alberts and et al. (1993). "Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease " Neurology 43(8): 1467-72.

Sawcer, S., H. B. Jones, D. Judge, F. Visser, A. Compston, P. N. Goodfellow and D. Clayton (1997). "Empirical genomewide significance levels established by whole genome simulations." Genet Epidemiol 14(3): 223-9.

Schipani, E., F. G. Hustmyer, C. Bergwitz and H. Jüppner (1994). "Polymorphism in exon M7 of the PTHR gene." Hum Mol Genet 3(7): 1210.

Schipani, E., H. Karga, A. C. Karaplis, J. T. Potts, Jr., H. M. Kronenberg, G. V. Segre, A. B. Abou-Samra and H. Jüppner (1993). "Identical complementary deoxyribonucleic acids encode a human renal and bone parathyroid hormone (PTH)/PTH-related peptide receptor." Endocrinology 132(5): 2157-65.

Schipani, E., K. Kruse and H. Jüppner (1995). "A constitutively active mutant PTH-PTHrP receptor in Jansen-type metaphyseal chondrodysplasia." Science 268(5207): 98-100.

Schipani, E., C. Langman, J. Hunzelman, M. Le Merrer, K. Y. Loke, M. J. Dillon, C. Silve and H. Jüppner (1999). "A novel parathyroid hormone (PTH)/PTH-related peptide receptor mutation in Jansen's metaphyseal chondrodysplasia." J Clin Endocrinol Metab 84(9): 3052-7.

Schipani, E., C. Langman, A. Parfitt, G. Jensen, S. Kikuchi, S. Kooh, W. Cole and H. Jüppner (1996). "Constitutively activated receptors for parathyroid hormone and parathyroid hormone-related peptide in Jansen's metaphyseal chondrodysplasia." N Engl J Med 335: 708-714.

Schipani, E., B. Lanske, J. Hunzelman, A. Luz, C. S. Kovacs, K. Lee, A. Pirro, H. M. Kronenberg and H. Jüppner (1997). "Targeted expression of constitutively active receptors for parathyroid hormone and parathyroid hormone-related peptide delays endochondral bone formation and rescues mice that lack parathyroid hormone-related peptide." Proc Natl Acad Sci U S A 94(25): 13689-94.

Schipani, E., L. S. Weinstein, C. Bergwitz, A. Iida-Klein, X. F. Kong, M. Stuhrmann, K. Kruse, M. P. Whyte, T. Murray, J. Schmidtke and et al. (1995). "Pseudohypoparathyroidism type Ib is not caused by mutations in the coding exons of the human parathyroid hormone (PTH)/PTH-related peptide receptor gene." J Clin Endocrinol Metab 80(5): 1611-21.

Seckinger, P., J. Klein-Nulend, C. Alander, R. C. Thompson, J. M. Dayer and L. G. Raisz (1990). "Natural and recombinant human IL-1 receptor antagonists block the effects of IL-1 on bone resorption and prostaglandin production." J Immunol 145(12): 4181-4.

Seeman, E. (1995). "The Dilemma of Osteoporosis in Men." Amer J Med 98(2A): 76S-88S.

Seeman, E (2001). "Sexual dimorphism in skeletal size, density and strength." J Clin Endocrinol Metab 86(10): 4576-84.

Seeman, E., Y. Duan, C. Fong and J. Edmonds (2001). "Fracture site-specific deficits in bone size and volumetric density in men with spine or hip fractures." J Bone Miner Res 16(1): 120-7.

Seeman, E., J. L. Hopper, L. A. Bach, M. E. Cooper, E. Parkinson, J. McKay and G. Jerums (1989). "Reduced bone mass in daughters of women with osteoporosis." N Engl J Med 320(9): 554-8.

Seeman, E., C. Tsalamandris, C. Formica, J. L. Hopper and J. McKay (1994). "Reduced femoral neck bone density in the daughters of women with hip fractures: the role of low peak bone density in the pathogenesis of osteoporosis." J Bone Miner Res 9(5): 739-43.

Sham, P. C. and D. Curtis (1995). "An extended transmission/disequilibrium test (TDT) for multi-allele marker loci." Ann Hum Genet 59(Pt 3): 323-36.

Sheffield, V., J. Beck, A. Kwitek, D. Sandstrom and E. Stone (1993). "The Sensitivity of Single-Strand Confrmation Polymorphism Analysis for the Detection of Single Base Substitutions." Genomics 16: 325-332.

Shioi, A., S. L. Teitelbaum, F. P. Ross, H. G. Welgus, H. Suzuki, J. Ohara and D. L. Lacey (1991). "Interleukin 4 inhibits murine osteoclast formation in vitro." J Cell Biochem 47(3): 272-7.

Shiraki, M., Y. Shiraki, C. Aoki, T. Hosoi, S. Inoue, M. Kaneki and Y. Ouchi (1997). "Association of bone mineral density with apolipoprotein E phenotype." J Bone Miner Res 12(9): 1438-45.

Silver, J., C. Yalcindag, A. Sela-Brown, R. Kilav and T. Naveh-Many (1999). "Regulation of the parathyroid hormone gene by vitamin D, calcium and phosphate." Kidney Int Suppl 73: S2-7.

Simmons, A., S. Barrington, M. O'Doherty and A. Coakley (1995). "Dual energy X-ray absorptiometry normal reference range use within the UK and the effect of different normal ranges on the assessment of bone density." Br J Radiol 68(812): 903-9.

Simonet, W. S., D. L. Lacey, C. R. Dunstan, M. Kelley, M. S. Chang, R. Luthy, H. Q. Nguyen, S. Wooden, L. Bennett, T. Boone, G. Shimamoto, M. DeRose, R. Elliott, A. Colombero, H. L. Tan, G. Trail, J. Sullivan, E. Davy, N. Bucay, L. Renshaw-Gegg, T. M. Hughes, D. Hill, W. Pattison, P. Campbell, W. J. Boyle and et al. (1997). "Osteoprotegerin: a novel secreted protein involved in the regulation of bone density " Cell 89(2): 309-19.

Slager, S. L. and V. J. Vieland (1997). "Investigating the numerical effects of ascertainment bias in linkage analysis: development of methods and preliminary results." Genet Epidemiol 14(6): 1119-24.

Slemenda, C. W., J. C. Christian, T. Reed, T. K. Reister, C. J. Williams and C. C. Johnston, Jr. (1992). "Long-term bone loss in men: effects of genetic and environmental factors." Ann Intern Med 117(4): 286-91.

Slemenda, C. W., J. C. Christian, C. J. Williams, J. A. Norton and C. C. Johnston (1991). "Genetic determinants of bone mass in adult women: a reevaluation of the twin model and the potential importance of gene interaction on heritability estimates." J Bone Miner Res 6(6): 561-567.

Smith, D. M., W. E. Nance, K. W. Kang, J. C. Christian and C. C. Johnston (1973). "Genetic factors in determining bone mass." J Clin Invest 52(11): 2800-8.

Smith, E., J. Boyd, G. Frank, H. Takahashi, R. Cohen, B. Specker, T. Williams, D. Lubahn and K. Korach (1995). "Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man." N Engl J Med 331: 1056-61.

Sobel, E. and K. Lange (1996). "Descent graphs in pedigree analysis: applications to haplotyping, location scores, and marker-sharing statistics." Am J Hum Genet 58(6): 1323-37.

Soroko, S., E. Barrett-Connor, S. Edelstein and D. Kritz-Silverstein (1994). "Family history of osteoporosis and bone mineral density at the axial skeleton: the Rancho Bernardo study." J Bone Miner Res 9(6): 761-769.

Sowers, M., M. Willing, T. Burns, S. Deschenes, B. Hollis, M. Crutchfield and M. Jannausch (1999). "Genetic Markers, Bone Mineral Density, and Serum Osteocalcin Levels." J Bone Miner Res 14(8): 1411-1419.

Sowers, M. R., M. Boehnke, M. L. Jannausch, M. Crutchfield, G. Corton and T. L. Burns. (1992). "Familiarity and partitioning the variability of femoral bone mineral density in women of child-bearing age." Calcif Tissue Int 50: 110-114.

Spector, T. D., R. W. Keen, N. K. Arden, N. A. Morrison, P. J. Major, T. V. Nguyen, P. J. Kelly, J. R. Baker, P. N. Sambrook, J. S. Lanchbury and et al. (1995). "Influence of vitamin D receptor genotype on bone mineral density in postmenopausal women: a twin study in Britain " Bmj 310(6991): 1357-60.

Spielman, R. S. and W. J. Ewens (1996). "The TDT and other family-based tests for linkage disequilibrium and association." Am J Hum Genet 59(5): 983-9.

Spielman, R. S. and W. J. Ewens (1998). "A sibship test for linkage in the presence of association: the sib transmission/disequilibrium test " Am J Hum Genet 62(2): 450-8.

Spielman, R. S., R. E. McGinnis and W. J. Ewens (1993). "Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM)." Am J Hum Genet 52(3): 506-16.

Spotila, L., J. Caminis, M. Devoto, K. Shimoya, L. Sereda, J. Ott, M. Whyte, A. Tenenhouse and D. Prockop (1996). "Osteopenia in 37 members of seven families: analysis based on a model of dominant inheritance." Molecular Medicine 2(3): 313-324.

Spotila, L., A. Colige and L. e. a. Sereda (1994). "Mutation analysis of coding sequences for type I procollagen in individuals with low bone density." J Bone Miner Res 9(6): 923-32.

Spotila, L., C. Constantinou, L. Sereda, A. Ganguly, B. Riggs and D. Prockop (1991). "Mutation in a gene for type I procollagen (COL1A2) in a woman with postmenopausal osteoporosis: evidence for phenotypic and genotypic overlap wtih mild osteogenesis imperfecta." Proc. Natl. Acad. Sci. USA 88: 5423-5427.

Staal, A., A. J. van Wijnen, J. C. Birkenhager, H. A. Pols, J. Prahl, H. DeLuca, M. P. Gaub, J. B. Lian, G. S. Stein, J. P. van Leeuwen and J. L. Stein (1996). "Distinct conformations of vitamin D receptor/retinoid X receptor-alpha heterodimers are specified by dinucleotide differences in the vitamin D-responsive elements of the osteocalcin and osteopontin genes." Mol Endocrinol 10(11): 1444-56.

Stein, B. and M. X. Yang (1995). "Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-kappa B and C/EBP beta." Mol Cell Biol 15(9): 4971-9.

Stewart, A., L. D. Calder, D. J. Torgerson, D.G. Seymour, L. D. Ritchie, C. P. Iglesias, and D. M. Reid (2000). "Prevalence of hip fracture risk factors in women aged 70 years and over." QJM 93(10): 677-80.

Strachan, T. and A. Read (1996). Human Molecular Genetics. Oxford, BIOS Scientific Publishers Ltd.

Strewler, G. (2000). "The physiology of Parathyroid Hormone-related Protein." New Engl J Med 342(3): 177-185.

Suarez, F., C. Rossignol and M. Garabedian (1998). "Interactive effect of estradiol and vitamin D receptor gene polymorphisms as a possible determinant of growth in male and female infants." J Clin Endocrinol Metab 83(10): 3563-8.

Suda, T., N. Udagawa, I. Nakamura, C. Miyaura and N. Takahashi (1995). "Modulation of osteoclast differentiation by local factors." Bone 17(2 Suppl): 87S-91S.

Sunthornthepvarakul, T., S. Churesigaew and S. Ngowngarmratana (1999). "A novel mutation of the signal peptide of the preproparathyroid hormone gene associated with autosomal recessive familial isolated hypoparathyroidism." J Clin Endocrinol Metab 84(10): 3792-6.

Sutherland, G. R., E. Baker, D. F. Callen, V. J. Hyland, G. Wong, S. Clark, S. S. Jones, L. K. Eglinton, M. F. Shannon, A. F. Lopez and et al. (1988). "Interleukin 4 is at 5q31 and interleukin 6 is at 7p15." Hum Genet 79(4): 335-7.

Sykes, B., D. Ogilvie and P. Wordsworth (1990). "Consistent linkage of dominantly inherited osteogenesis imperfecta to the type I collagen loci." Am J Hum Genet 46: 293-307.

Tabensky, A. D., J. Williams, V. DeLuca, E. Briganti and E. Seeman (1996). "Bone mass, areal, and volumetric bone density are equally accurate, sensitive, and specific surrogates of the breaking strength of the vertebral body: an in vitro study." J Bone Miner Res 11(12): 1981-8.

Tabensky, A., Y. Duan, J. Edmonds and E. Seeman (2001). "The contribution of reduced peak accrual of bone and age-related bone loss to osteoporosis at the spine and hip: insights from the daughters of women with vertebral or hip fractures." J Bone Miner Res 16(6): 1101-7.

Taboulet, J., M. Frenkian, J. L. Frendo, N. Feingold, A. Jullienne and M. C. de Vernejoul (1998). "Calcitonin receptor polymorphism is associated with a decreased fracture risk in post-menopausal women." Hum Mol Genet 7(13): 2129-33.

Taguchi, Y., M. Yamamoto, T. Yamate, S. C. Lin, H. Mocharla, P. DeTogni, N. Nakayama, B. F. Boyce, E. Abe and S. C. Manolagas (1998). "Interleukin-6-type cytokines stimulate mesenchymal progenitor differentiation toward the osteoblastic lineage." Proc Assoc Am Physicians 110(6): 559-74.

Tai, Y., E. Duncan, J. Wass, M. Brown and J. Sinsheimer (2001). "The Effect of Covariate Adjustments in Linnkage Studies of Bone Mineral Density." in preparation.

Takacs, I., D. L. Koller, M. Peacock, J. C. Christian, W. E. Evans, S. L. Hui, P. M. Conneally, C. C. Johnston, Jr., T. Foroud and M. J. Econs (2000). "Sib pair linkage and association studies between bone mineral density and the interleukin-6 gene locus." Bone 27(1): 169-73.

Takacs, I., D. L. Koller, M. Peacock, J. C. Christian, S. L. Hui, P. M. Conneally, C. C. Johnston, Jr., T. Foroud and M. J. Econs (1999). "Sibling pair linkage and association studies between bone mineral density and the insulin-like growth factor I gene locus [see comments] [published erratum appears in J Clin Endocrinol Metab 2000 Jan;85(1):138]." J Clin Endocrinol Metab 84(12): 4467-71.

Takai, H., M. Kanematsu, K. Yano, E. Tsuda, K. Higashio, K. Ikeda, K. Watanabe and Y. Yamada (1998). "Transforming growth factor-beta stimulates the production of osteoprotegerin/osteoclastogenesis inhibitory factor by bone marrow stromal cells." J Biol Chem 273(42): 27091-6.

Takasu, H., J. Guo and F. R. Bringhurst (1999). "Dual signaling and ligand selectivity of the human PTH/PTHrP receptor." J Bone Miner Res 14(1): 11-20.

Tamura, T., N. Udagawa, N. Takahashi, C. Miyaura, S. Tanaka, Y. Yamada, Y. Koishihara, Y. Ohsugi, K. Kumaki, T. Taga and et al. (1993). "Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6." Proc Natl Acad Sci U S A 90(24): 11924-8.

Tatusova, T. and T. Madden (1999). "Blast 2 sequences – a new tool for comparing protein and nucleotide sequences." FEMS Microbiol Lett. 174: 247-250.

Taylor, P., K. Munson and D. Gjerde (1998). Detection of mutations and polymorphisms on the WAVE DNA Fragment Analysis System, Transgenomic Inc.

Templeton, A. R., A. G. Clark, K. M. Weiss, D. A. Nickerson, E. Boerwinkle and C. F. Sing (2000). "Recombinational and mutational hotspots within the human lipoprotein lipase gene." Am J Hum Genet 66(1): 69-83.

Terai, K., T. Takano-Yamamoto and Y. e. a. Ohba (1999). "Role of osteopontin inboen remodeling caused by mechanical stress." J Bone Miner Res 14(6): 839-49.

Terwilliger, J. D. and J. Ott (1992). "A haplotype-based 'haplotype relative risk' approach to detecting allelic associations." Hum Hered 42(6): 337-46.

Terwilliger, J. D., W. D. Shannon, G. M. Lathrop, J. P. Nolan, L. R. Goldin, G. A. Chase and D. E. Weeks (1997). "True and false positive peaks in genomewide scans: applications of length-biased sampling to linkage mapping " Am J Hum Genet 61(2): 430-8.

Terwilliger, J. D. and K. M. Weiss (1998). "Linkage disequilibrium mapping of complex disease: fantasy or reality?" Curr Opin Biotechnol 9(6): 578-94.

Thomson, B., J. Saklatvala and T. Chambers (1986). "Osteoblasts mediate interleukin 1 stimulation of bone resorption by rat osteoclasts." J Exp Med 164: 104-112.

Thomson, B. M., G. R. Mundy and T. J. Chambers (1987). "Tumor necrosis factors alpha and beta induce osteoblastic cells to stimulate osteoclastic bone resorption." J Immunol 138(3): 775-9.

Todd, J. A. (1995). "Genetic analysis of type 1 diabetes using whole genome approaches." Proc Natl Acad Sci USA 92(19): 8560-5.

Tokita, A., P. J. Kelly, T. V. Nguyen, J. C. Qi, N. A. Morrison, L. Risteli, J. Risteli, P. N. Sambrook and J. A. Eisman (1994). "Genetic influences on type I collagen synthesis and degradation: further evidence for genetic regulation of bone turnover." J Clin Endocrinol Metab 78(6): 1461-6.

Tsuji, S., B. Munkhbat, M. Hagihara, I. Tsuritani, H. Abe and K. Tsuji (1998). "HLA-A*24-B*07-DRB1*01 haplotype implicated with genetic disposition of peak bone mass in healthy young Japanese women." Hum Immunol 59(4): 243-9.

Tsukamoto, K., H. Orimo, T. Hosoi, M. Miyao, N. Ota, T. Nakajima, H. Yoshida, S. Watanabe, T. Suzuki and M. Emi (2000). "Association of bone mineral density with polymorphism of the human calcium-sensing receptor locus." Calcif Tissue Int 66(3): 181-3.

Tsukamoto, K., H. Yoshida, S. Watanabe, T. Suzuki, M. Miyao, T. Hosoi, H. Orimo and M. Emi (1999). "Association of radial bone mineral density with CA repeat polymorphism at the interleukin 6 locus in postmenopausal Japanese women." J Hum Genet 44(3): 148-51.

Tsukii, K., N. Shima, S. Mochizuki, K. Yamaguchi, M. Kinosaki, K. Yano, O. Shibata, N. Udagawa, H. Yasuda, T. Suda and K. Higashio (1998). "Osteoclast differentiation factor mediates an essential signal for bone resorption induced by 1 alpha,25-dihydroxyvitamin D3, prostaglandin E2, or parathyroid hormone in the microenvironment of bone." Biochem Biophys Res Commun 246(2): 337-41.

Udagawa, N., N. Takahashi, E. Jimi, K. Matsuzaki, T. Tsurukai, K. Itoh, N. Nakagawa, H. Yasuda, M. Goto, E. Tsuda, K. Higashio, M. T. Gillespie, T. J. Martin and T. Suda (1999). "Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor: receptor activator of NF-kappa B ligand." Bone 25(5): 517-23.

Udagawa, N., N. Takahashi, T. Katagiri, T. Tamura, S. Wada, D. M. Findlay, T. J. Martin, H. Hirota, T. Taga, T. Kishimoto and et al. (1995). "Interleukin (IL)-6 induction of osteoclast differentiation depends on IL-6 receptors expressed on osteoblastic cells but not on osteoclast progenitors." J Exp Med 182(5): 1461-8.

Ueno, K., T. Katayama, T. Miyamoto and Y. Koshihara (1992). "Interleukin-4 enhances in vitro mineralization in human osteoblast-like cells." Biochem Biophys Res Commun 189(3): 1521-6.

Uitterlinden, A., H. Burger, Q. Huang, F. Yue, F. McGuigan, S. Grant, A. Hofman, J. van Leeuwen, H. Pols and S. Ralston (1998). "Relation of alleles of the collagen type I alpha 1 gene to bone density and the risk of osteoporotic fractures in postmenopausal women." N Engl J Med 338(15): 1016-1021.

Uitterlinden, A. G., H. Burger, C. M. van Duijn, Q. Huang, A. Hofman, J. C. Birkenhager, J. P. van Leeuwen and H. A. Pols (2000). "Adjacent genes, for COL2A1 and the vitamin D receptor, are associated with separate features of radiographic osteoarthritis of the knee." Arthritis Rheum 43(7): 1456-64.

Uitterlinden, A. G., H. A. Pols, H. Burger, Q. Huang, P. L. Van Daele, C. M. Van Duijn, A. Hofman, J. C. Birkenhager and J. P. Van Leeuwen (1996). "A large-scale population-based study of the association of vitamin D receptor gene polymorphisms with bone mineral density." J Bone Miner Res 11(9): 1241-8.

Uitterlinden, A. G., A. E. Weel, H. Burger, Y. Fang, C. M. van Duijn, A. Hofman, J. P. van Leeuwen and H. A. Pols (2001). "Interaction between the vitamin D receptor gene and collagen type Ialpha1 gene in susceptibility for fracture." J Bone Miner Res 16(2): 379-85.

Usdin, T. B., T. I. Bonner, G. Harta and E. Mezey (1996). "Distribution of parathyroid hormone-2 receptor messenger ribonucleic acid in rat." Endocrinology 137(10): 4285-97.

Usdin, T. B., C. Gruber and T. I. Bonner (1995). "Identification and functional expression of a receptor selectively recognizing parathyroid hormone, the PTH2 receptor." J Biol Chem 270(26): 15455-8.

Vandevyver, C., J. Vanhoof, K. Declerck, P. Stinissen, C. Vandervorst, L. Michiels, J. J. Cassiman, S. Boonen, J. Raus and P. Geusens (1999). "Lack of association between estrogen receptor genotypes and bone mineral density, fracture history, or muscle strength in elderly women." J Bone Miner Res 14(9): 1576-82.

Vidal, N. O., H. Brandstrom, K. B. Jonsson and C. Ohlsson (1998). "Osteoprotegerin mRNA is expressed in primary human osteoblast-like cells: down-regulation by glucocorticoids." J Endocrinol 159(1): 191-5.

Vidal, O., L. G. Kindblom and C. Ohlsson (1999). "Expression and localization of estrogen receptor-beta in murine and human bone." J Bone Miner Res 14(6): 923-9.

Wasnich, R. (1993). "Bone mass measurement: prediction of risk." Am J Med 95(5A): 6S-10S.

Waters, K. and T. Spelsberg (1999). Gonadal steroids and receptors. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. M. Favus. Philadelphia, Lippincott, Williams & Wilkins: 104-110.

Weeks, D. and K. Lange (1988). "The affected-pedigree-member method of linkage analysis." Am J Hum Genet 42: 315-326.

Weinstein, R. S., R. L. Jilka, A. M. Parfitt and S. C. Manolagas (1997). "The effects of androgen deficiency on murine bone remodeling and bone mineral density are mediated via cells of the osteoblastic lineage." Endocrinology 138(9): 4013-21.

Weir, E. C., W. M. Philbrick, M. Amling, L. A. Neff, R. Baron and A. E. Broadus (1996). "Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation." Proc Natl Acad Sci U S A 93(19): 10240-5.

Wijsman, I. and C. Amos (1997). "Genetic analysis of simulated oligogenic traits in nuclear and extended pedigress: summary of GAW10 contributions." Genet. Epidemiol. 14: 719-735.

Williams, J. T. and J. Blangero (1999). "Comparison of variance components and sibpair-based approaches to quantitative trait linkage analysis in unselected samples." Genet Epidemiol 16(2): 113-34.

Willing, M., M. Sowers, D. Aron, M. K. Clark, T. Burns, C. Bunten, M. Crutchfield, D. D'Agostino and M. Jannausch (1998). "Bone mineral density and its change in white women: estrogen and vitamin D receptor genotypes and their interaction." J Bone Miner Res 13(4): 695-705.

Wishart, J. M., M. Horowitz, A. G. Need, F. Scopacasa, H. A. Morris, P. M. Clifton and B. E. Nordin (1997). "Relations between calcium intake, calcitriol, polymorphisms of the vitamin D receptor gene, and calcium absorption in premenopausal women." Am J Clin Nutr 65(3): 798-802.

Witte, J. S., R. C. Elston and N. J. Schork (1996). "Genetic dissection of complex traits." Nat Genet 12(4): 355-6.

Woitge, H. and J. Markus (2000). Risk assessment of osteoporosis II: biochemical markers of bone turnover: bone resorption indices. Osteoporosis and Metabolic Bone Diseases. C. Rosen. Philadelphia, WB Saunders. 20: 503-525.

Wong, B. R., J. Rho, J. Arron, E. Robinson, J. Orlinick, M. Chao, S. Kalachikov, E. Cayani, F. S. Bartlett, 3rd, W. N. Frankel, S. Y. Lee and Y. Choi (1997). "TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells." J Biol Chem 272(40): 25190-4.

Woodhouse, A. and D. Black (2000). "BMD at various sites for the prediction of hip fracture." J Bone Miner Res 2000(Suppl 1): S145.

Wordsworth, P. (1995). "Genes and arthritis." Br Med Bull 51(2): 249-66.

Wright, F. (1997). "Teh phenotypic difference discards sib-pair QTL linkage information." Am J Hum Genet 60(3): 740-742.

Wu, C., C. Gluer, Y. Lu, T. Fuerst, D. Hans and H. K. Genant (1998). "Ultrasound characterization of bone demineralization." Calcif Tissue Int 62(2): 133-9.

Wysolmerski, J. and A. Broadus (1994). "Hypercalcaemia of malignancy: the central role of parathyroid hormone-related protein." Annu Rev Med 45: 189-200.

Xu, T., P. Bianco, L. W. Fisher, G. Longenecker, E. Smith, S. Goldstein, J. Bonadio, A. Boskey, A. M. Heegaard, B. Sommer, K. Satomura, P. Dominguez, C. Zhao, A. B. Kulkarni, P. G. Robey and M. F. Young (1998). "Targeted disruption of the biglycan gene leads to an osteoporosis-like phenotype in mice." Nat Genet 20(1): 78-82.

Yamada, Y., A. Harada, T. Hosoi, A. Miyauchi, K. Ikeda, H. Ohta and M. Shiraki (2000). "Association of transforming growth factor beta1 genotype with therapeutic response to active vitamin D for postmenopausal osteoporosis." J Bone Miner Res 15(3): 415-20.

Yamada, Y., A. Miyauchi, J. Goto, Y. Takagi, H. Okuizumi, M. Kanematsu, M. Hase, H. Takai, A. Harada and K. Ikeda (1998). "Association of a polymorphism of the transforming growth factor-beta1 gene with genetic susceptibility to osteoporosis in postmenopausal Japanese women." J Bone Miner Res 13(10): 1569-76.

Yamagata, Z., T. Miyamura, S. Iijima, A. Asaka, M. Sasaki, J. Kato and K. Koizumi (1994). "Vitamin D receptor gene polymorphism and bone mineral density in healthy Japanese women." Lancet 344(8928): 1027.

Yasuda, H., N. Shima, N. Nakagawa, S. I. Mochizuki, K. Yano, N. Fujise, Y. Sato, M. Goto, K. Yamaguchi, M. Kuriyama, T. Kanno, A. Murakami, E. Tsuda, T. Morinaga and K. Higashio (1998).

"Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro." Endocrinology 139(3): 1329-37.

Yasuda, H., N. Shima, N. Nakagawa, K. Yamaguchi, M. Kinosaki, S. Mochizuki, A. Tomoyasu, K. Yano, M. Goto, A. Murakami, E. Tsuda, T. Morinaga, K. Higashio, N. Udagawa, N. Takahashi and T. Suda (1998). "Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL." Proc Natl Acad Sci U S A 95(7): 3597-602.

Yin, T., T. Taga, M. L. Tsang, K. Yasukawa, T. Kishimoto and Y. C. Yang (1993). "Involvement of IL-6 signal transducer gp130 in IL-11-mediated signal transduction." J Immunol 151(5): 2555-61.

Yiu, G., W. Gu and N. Hecht (1994). "Heterogeneity in the 5' untranslated region of mouse cytochrome CT leads to an altered translational status of the mRNAs." Nucleic Acids Res 22: 4599-4606.

Yokota, T., T. Otsuka, T. Mosmann, J. Banchereau, T. DeFrance, D. Blanchard, J. E. De Vries, F. Lee and K. Arai (1986). "Isolation and characterization of a human interleukin cDNA clone, homologous to mouse B-cell stimulatory factor 1, that expresses B-cell- and T-cell-stimulating activities." Proc Natl Acad Sci U S A 83(16): 5894-8.

Yoshida, H., S. Hayashi, T. Kunisada, M. Ogawa, S. Nishikawa, H. Okamura, T. Sudo and L. D. Shultz (1990). "The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene." Nature 345(6274): 442-4.

Yoshitake, H., S. R. Rittling, D. T. Denhardt and M. Noda (1999). "Osteopontin-deficient mice are resistant to ovariectomy-induced bone resorption [published erratum appears in Proc Natl Acad Sci U S A 1999 Sep 14;96(19):10944]." Proc Natl Acad Sci U S A 96(14): 8156-60.

Yoshizawa, T., Y. Handa, Y. Uematsu, S. Takeda, K. Sekine, Y. Yoshihara, T. Kawakami, K. Arioka, H. Sato, Y. Uchiyama, S. Masushige, A. Fukamizu, T. Matsumoto and S. Kato (1997). "Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning." Nat Genet 16(4): 391-6.

Zhang, P., A. S. Jobert, A. Couvineau and C. Silve (1998). "A homozygous inactivating mutation in the parathyroid hormone/parathyroid hormone-related peptide receptor causing Blomstrand chondrodysplasia." J Clin Endocrinol Metab 83(9): 3365-8.

Zmuda, J., J. Cauley, M. Danielson, T. Theobald and R. Ferrell (1999). "Vitamin D receptor translation initiation codon polymorphism and markers of osteoporotic risk in older African-American women." Osteoporosis Int 9: 214-219.

Zmuda, J. M., J. A. Cauley, M. E. Danielson, R. L. Wolf and R. E. Ferrell (1997). "Vitamin D receptor gene polymorphisms, bone turnover, and rates of bone loss in older African-American women." J Bone Miner Res 12(9): 1446-52.