



LUND UNIVERSITY

Osteoporosis in elderly women; Bone traits, fracture and the PTH gene complex

Tenne, Max

2011

[Link to publication](#)

Citation for published version (APA):

Tenne, M. (2011). *Osteoporosis in elderly women; Bone traits, fracture and the PTH gene complex*. [Doctoral Thesis (compilation), Orthopedics]. Clinical and Molecular Osteoporosis Research Unit, Clinical Sciences, Malmö.

Total number of authors:

1

General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

**Clinical and Molecular Osteoporosis Research Unit
Clinical Sciences, Malmö
Department of Orthopaedic Surgery, Malmö**

On osteoporosis in elderly women

**Bone traits, fracture and the PTH gene
complex**

**Max Tenne
MD**



LUND UNIVERSITY
Faculty of Medicine

Opponent Professor Lars Nordsletten, Oslo

Supervisor Professor Kristina Åkesson

Doctoral Dissertation Series 2011:100

© Max Tenne 2011
and the copyright owners of paper I-IV
Max.Tenne@skane.se
Printed by Media-Tryck, Lund University, Lund, Sweden
Lund University, Faculty of Medicine
Doctoral Dissertation Series 2011:100

ISSN 1652-8220
ISBN 978-91-86871-50-5

To my wife and daughter

Contents

List of original publications	7
Abbreviations	8
Introduction	9
Review of the literature	11
Osteoporosis, fragility fractures and bone biology	11
Skeletal genetics	17
Techniques for assessment of bone mass.....	23
Bone geometry and biomechanical properties	25
Aims of the study	27
Subjects and methods	28
Study participants and assessment of data	28
Genotyping	29
Bone mineral density assessment	32
Hip strength analysis	33
Visual assessment of the DXA image	33
Statistical methods	35

Results	37
Study I	37
Study II	38
Study III	40
Study IV	41
Discussion	43
Conclusions and future prospects	49
Acknowledgements	50
Sammanfattning på Svenska	52
References	55
Original publications	65

List of original publications

I

M. Tenne, F. McGuigan, L. Jansson, P. Gerdhem, K. Obrant, H. Luthman, K. Åkesson. Genetic variation in the PTH pathway and bone phenotypes in elderly women: Evaluation of *PTH*, *PTH LH*, *PTH R1* and *PTH R2* genes. *Bone* 42 (2008) 719-727

II

M. Tenne, F. McGuigan, H. Ahlborg, P. Gerdhem, K. Åkesson. Variation in the *PTH* Gene, Hip Fracture, and Femoral Neck Geometry in Elderly Women. *Calcif Tissue Int* (2010) 86:359-366

III

M. Tenne, F. McGuigan, J. Besjakov, P. Gerdhem, K. Åkesson. Degenerative Changes at the Lumbar Spine – Implications for Bone Mineral Density Measurement in Elderly Women. Submitted.

IV

M. Tenne, P. Gerdhem, K. Obrant, H. Luthman, K. Åkesson, F. McGuigan. Relationship between *PTH* pathway genes, vertebral size and vertebral degeneration in elderly women. In manuscript.

Abbreviations

ALP	Alkaline phosphatase
BMD	Bone mineral density
BMI	Body mass index
BstBI	Bacillus stearothermophilus B225 (source of restriction enzyme)
BUA	Broadband ultrasound attenuation
CaSR	Calcium sensing receptor
CSMI	Cross section moment of inertia
DraII	Deinococcus radiophilus (source of restriction enzyme)
DXA	Dual X-ray absorptiometry
HSA	Hip strength analysis
HWE	Hardy Weinberg equilibrium
IGF-1	Insulin like growth factor 1
IL-6	Interleukin-6
LD	Linkage disequilibrium
LOD	Linkage of disease
PTH	Parathyroid hormone
<i>PTH</i>	Parathyroid hormone gene
PTH LH	PTH like hormone
PTHrP	PTH related peptide
<i>PTH LH</i>	PTH like hormone gene
PTH R1	PTH receptor 1
<i>PTH 1 R</i>	PTH receptor 1 gene
PTH R2	PTH receptor 2
<i>PTH 2 R</i>	PTH receptor 2 gene
1,25(OH) ₂ D	1,25-dihydroxyvitamin D
25OHD	25-hydroxyvitamin D
SM	Section modulus
SNP	Single nucleotide polymorphism
SOS	Speed of sound
VDR	Vitamin D receptor

Introduction

Osteoporosis is a common skeletal disease characterized by low bone mineral density (BMD) and micro-architectural deterioration of bone tissue. The ultimate outcome of osteoporosis is a fragility fracture, in Sweden approximately 70 000 per year. The definition of osteoporosis is a bone mass below -2.5 standard deviations (SD) compared to young adults measured with dual energy X-ray absorptiometry (DXA). The DXA technique is the gold standard for BMD measurements but not in all aspects ideal. The results from lumbar spine scans are, particularly in the elderly, often distorted and misleadingly elevated, due to degenerative changes, surgical implants, fractures and vascular calcifications.

Parathyroid hormone (PTH) is a key regulator of calcium metabolism, maintaining the calcium ion levels in serum by modulating osteoclastic bone resorption and calcium reabsorption in the kidneys, whereas parathyroid hormone-like hormone (PTHrP) contributes to skeletal development through regulation of chondrocyte proliferation and differentiation during early bone growth. PTH and PTHrP act through the same receptor, the PTH/PTHrP receptor (PTHR1). A second receptor, the PTH receptor 2 (PTHR2) has been identified however comparatively little is known about its biological importance. Constitutive PTH hyper-secretion induces bone resorption as seen in hyperparathyroidism, whereas intermittent PTH administration is a potent bone anabolic agent, stimulating osteoblast proliferation and differentiation. PTH and PTHrP are furthermore known to influence both bone size and structure.

There are well known non genetic life style factors contributing to bone loss such as smoking, inactivity and dietary deficiencies but the heritability of bone related phenotypes is reported to be up to 80%. Osteoporosis and bone strength are however complex traits and are under polygenic regulation.

Osteoporosis is a disease which develops over decades and prevention of fracture is dependent on early detection. We have addressed two different strands towards earlier diagnosis:

Firstly by trying to identify individuals with a genetic predisposition to osteoporosis; *PTH* was our candidate gene since the PTH hormone is the main calcium ion regulator and both PTH and the PTH like hormone appear to have direct effects on the size and micro-architecture of the skeleton.

Secondly by trying to improve the clinical interpretation of DXA scans through identifying individuals and individual vertebrae with lumbar degenerative changes. These manifestations falsely elevate the DXA results, delay diagnosis and hamper studies aiming to identify the importance of other risk factors for spinal bone loss.

To answer these questions we used data from the Malmö Osteoporosis Prospective Risk Assessment (OPRA) study which comprises over 1000 women, all 75 years at inclusion. These women were extensively investigated over 10 years with DXA measurements, blood samples and registration of life style factors and fractures.

Review of the literature

Osteoporosis, fragility fractures and bone biology

Osteoporosis, definition and prevalence

The English surgeon, Sir Astley Cooper, described osteoporosis over 150 years ago. He noted a relation between hip fractures in the elderly and reduced bone mass or bone quality [1]. The association between estrogen deficiency and bone loss in postmenopausal women was discovered in 1940 by the American endocrinologist, Fuller Albright [2].

Today we define osteoporosis as loss of bone mass and micro architectural deterioration of the bone tissue with subsequent bone fragility and increased fracture risk [3]. In 1994 the World Health Organization, WHO, published an operational definition of osteoporosis: a BMD value of -2.5 standard deviations (SD) or lower compared to young adults measured with DXA technique. If BMD is below -2.5 SD and a fragility fracture also is prevalent the diagnosis is upgraded to established osteoporosis. Osteopenia, the pre-disease state of osteoporosis, is defined as BMD above -2.5 SD but below -1 SD. A BMD value above -1 SD is considered within the normal variation of bone mass [4]. The risk of sustaining a fragility fracture approximately doubles with each SD reduction in the T-score of BMD, 2.6 times the risk for fracture of the femoral neck and 2.3 times for vertebral fracture [5, 6]. The T-score represents BMD compared to healthy young adults and is often presented alongside with the Z-score where BMD is compared to age matched individuals.

Osteoporosis is classified as primary or secondary osteoporosis and the primary type into postmenopausal (type I) or senile osteoporosis (type II). Postmenopausal osteoporosis primarily affects trabecular bone and is related to the reduced estrogen levels after menopause. Senile osteoporosis is characterized of by both cortical thinning and trabecular bone loss and is

prevalent in both men and women at older ages. Secondary osteoporosis also affects both cortical and trabecular bone and is related to a number of conditions, for example hyperparathyroidism, anorexia nervosa, renal failure, Cushing's disease and glucocorticoid substitution [7].

Hyperparathyroidism can be primary or secondary to other disease affecting Ca serum levels. Primary hyperparathyroidism is prevalent in 3-4% of all postmenopausal women in Sweden, and is often asymptomatic for many years. In the skeleton it mainly causes cortical bone loss [8]. It is controversial how long the disease should be monitored before medical or surgical intervention [9].

Pathogenesis

Primary osteoporosis is a multifactorial disease where about 70% of contributing factors are genetic and 30% environmental [10]. These factors influence the equilibrium of the bone remodeling process resulting in bone resorption that exceeds bone formation [2]. In the first years after menopause the bone loss rate can be up to 2-5% per year [11]. Losing bone mass also contributes to a structural problem when the trabecular microstructure deteriorate, therefore contributing to increased fragility and fracture risk [12]. There is strong evidence for an association between a high rate of bone remodeling and osteoporosis [13]. The rate of bone remodeling can be assessed by measuring biochemical markers. These are classified as either bone formation or resorption markers. Alkaline phosphatase (ALP) and osteocalcin are examples of bone formation markers. Hydroxyproline and deoxypyridinoline reflecting collagen breakdown are examples of bone resorption markers [14, 15].

Risk factors for osteoporosis and fracture

Vulnerability to osteoporosis and subsequent fracture risk depends on many factors affecting peak bone mass, bone remodeling, and predisposition to fall. The most important risk factors are low BMD and high age in both women and men [16]. The risk factors can be divided into those predicting low bone mass and those predicting fracture independent of bone mass. Female sex, Caucasian or Asian ethnic origin, premature menopause, calcium or vitamin D deficiency and immobilization are examples of

predictors of low BMD. Age, fracture history, poor visual acuity, neuromuscular disorders, smoking and low body weight are predictors of fracture independent of BMD [17]. There are several scores where these risk factors, in addition to BMD, are added up to enable prediction of fracture and thus aiding treatment decisions [18]. WHO has recently developed the widely known fracture risk assessment tool, FRAX®, where the 10 year probability for hip or other major osteoporotic fracture (clinical spine, forearm, hip or shoulder fracture) is calculated [19].

Fragility fractures

Fractures related to osteoporosis are in general located to the hip, pelvis, distal radius, proximal humerus and spine. The hip fracture has the highest impact on quality of life and is associated with high morbidity and mortality [20]. Less than 50% of these patients regain their level of activity as before the hip fracture after 1 year [21]. Vertebral fractures are also a common cause of morbidity with a prevalence of 15-20% in postmenopausal women and often associated with significant prolonged pain [22, 23]. The vertebral fractures are however in general not related to fall and in many cases not diagnosed unless radiological screening is performed [24]. The incidence of distal radius fractures begins to increase already at 45 years of age in women and reaches a plateau at 60 years [25].

Half of the female population over 50 years of age in Sweden will suffer from a fragility fracture [26]. The incidence and prevalence is equally high in the rest of Scandinavia and the United Kingdom. Worldwide it is estimated that one in three women and one in five men that will suffer a fragility fracture after the age of 50 years and the one-in-six risk for a white women sustaining a hip fracture exceeds the one-in-nine risk for breast cancer [27]. Alongside the suffering of the fracture patient, osteoporosis and subsequent fractures are a major burden to healthcare costs worldwide [28]. In 2004 for example the estimated cost of treating hip fractures alone was 25 billion euros yearly in Europe [29].

Prevention of fracture

Prevention of fracture involves nutrition, medication and life style factors.

The association between low dietary calcium intake and fracture is inconsistent [30]. Nevertheless, meta-analysis shows a preventive effect of calcium supplementation (in combination with and without vitamin D) on fragility fracture in people over 50 years [31]. Vitamin D has in addition to its positive effect on bone also a role in promoting growth of skeletal muscle fibers subsequently reducing risk for falls and fracture [32, 33].

When osteoporosis or a fragility fracture is prevalent the first choice of treatment is with bisphosphonates which have the most extensively documented effect. Bisphosphonates interfere with the osteoclasts and inhibit bone resorption. The fracture preventing effect is greatest in vertebrae (30-50% risk reduction) and lesser in the peripheral skeleton [34]. Bisphosphonates can be administered per os weekly or with one infusion yearly. Side effects include upper gastrointestinal injury and rarely, osteonecrosis of the jaw [35] or atypical hip fracture [36]. An alternative is strontium ranelate where strontium is an element that resembles calcium and, in a manner which is not yet fully understood, hampers bone resorption. It is also believed to have a small anabolic effect and is more effective in preventing hip than vertebral fractures [37]. For patients with severe osteoporosis teriparatide (PTH 1-34) or the intact PTH (1-84) has a powerful anabolic effect on bone tissue when administered as daily injections. It also restores bone architecture and increases bone strength to a higher extent compared with anti-resorptive treatment. After 18 months treatment a 9-13% increase was seen with teriparatide and 7% with PTH 1-84 in vertebral BMD in large cohorts [38, 39]. Fracture risk reduction for vertebral fractures reaching above fifty percent, again with less pronounced effects on non-vertebral fractures. The latest registered drug for osteoporosis is denosumab which is a monoclonal antibody designed to target RANKL (receptor activator of nuclear factor kappa-B ligand). RANKL is a key factor for osteoclast differentiation and activation. Denosumab is administered as injections twice yearly and the fracture preventing effect is comparable with teriparatide [40].

Among life style factors there are several meta-analyses confirming the negative effect of smoking, especially on hip fractures in both men and women [41]. Kanis et al have furthermore showed that alcohol intake exceeding 2 units per day also increased the risk for fracture [42]. Low body mass index, BMI, (below 20 kg/m²) is moreover associated with

increased fracture risk [16] and physical activity, especially during growth and among the elderly, is related to lower risk [43, 44].

Calcium homeostasis and PTH

Strict calcium (Ca) homeostasis is essential for the human organism. The biologically active ionized calcium varies in serum within a very narrow span (1.15-1.35 mmol/l) and controls important functions such as neurotransmission, muscle contraction, blood clotting and hormone secretion. The main regulator of serum Ca is PTH, secreted from the parathyroid glands and controlled by a tight feedback system. PTH acts via the PTH type 1 receptor (PTH1R) a class B G-protein-coupled receptor with seven trans-membrane domains mainly present bone, kidney and intestine [45]. Vitamin D and to some extent calcitonin (in children) are also involved in calcium homeostasis.

A few percent reduction of s-Ca is enough for the Ca sensing receptors (CaSR) in the parathyroid glands to induce a large increase in PTH secretion [46]. In the kidneys, PTH acts directly on the tubules, increasing re-absorption of calcium and also inducing conversion of 25-hydroxy vitamin D (25OHD) into the active metabolite 1,25(OH)₂ vitamin D (1,25(OH)₂D) [47]. PTH and the active vitamin D is then both increasing serum Ca via their respective receptors in bone, intestine and kidney. The effect in bone tissue is increased bone resorption mediated through increased osteoclastic activity [48]. In the intestine PTH is suggested to facilitate Ca uptake through a direct mechanism [49].

When serum Ca concentration increases both Ca and vitamin D function as a negative feedback mechanism on the parathyroid glands and decrease PTH secretion [50], fig 1.

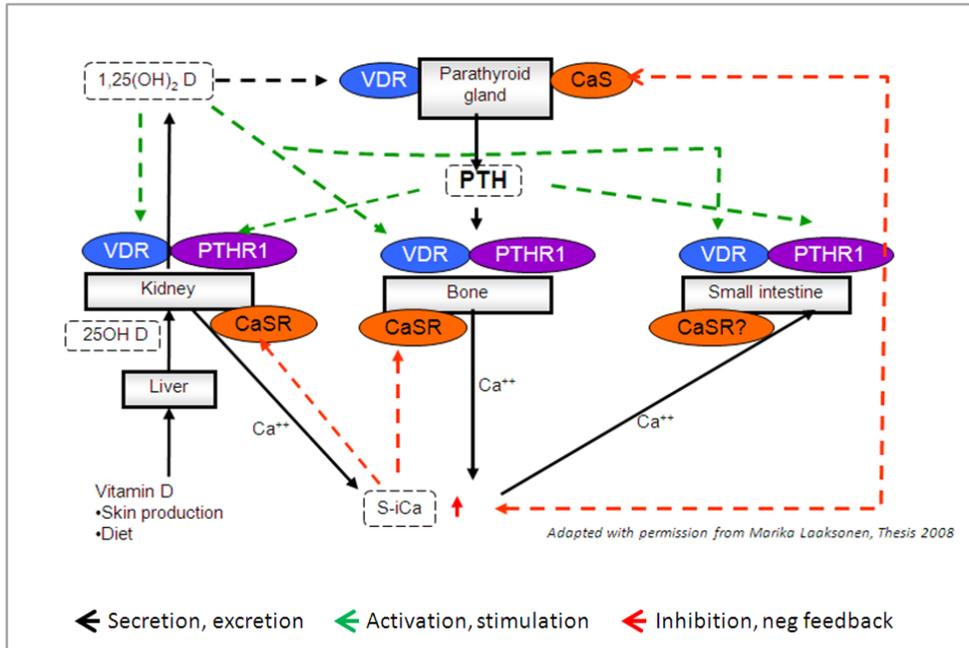


Figure 1. The feedback system of calcium homeostasis where PTH is the main regulating hormone in conjunction with Vitamin D

The PTH like hormone (PTH_{LH}) is not directly involved in the calcium homeostasis but rather in bone growth in children through regulation of chondrocyte proliferation and differentiation [51]. PTH_{LH} is also seen in cancer patients as a para-malignant phenomenon and in this case causing hypercalcemia [52]. PTH receptor 2 (PTH_{R2}) is activated by PTH but not PTH_{LH}. PTH_{R2} is present in brain and pancreas but the function is largely unknown [53]. Genetic variation in the *PTH2R* gene has been associated with familial osteoarthritis [54, 55]

Skeletal genetics

Several lines of evidence, from twin studies, mother daughter studies and family studies indicate that genetic factors play an important role in the pathogenesis of osteoporosis and osteoporosis inheritance describes a pattern which is consistent with modest effects from many genes rather than large effects from a few genes [56]. Consequently, detecting the effect of a single gene on BMD or other components of bone strength are difficult even in large study cohorts. Furthermore, the complex nature of the disease, which involves interactions between genetics, age, metabolic and a multitude of environmental factors, make it challenging to dissect out the contribution of each of these components.

Heritability – Bone mineral density

Twin and family studies show a heritability (i.e. the proportion of the variance which is genetic) of 50-85% for accrual of peak bone mass, which is dependent on sex, age and skeletal site. For example, when BMD correlations within monozygotic and dizygotic twin pairs are investigated there is evidence for heritability being higher for spinal BMD compared to hip and forearm BMD. A recent Finnish study comprising 300 men shows 83% heritability in BMD of the lumbar spine and 75% at the femoral neck [57]. Heritability, for some phenotypes at least, may vary with age although there is evidence both for and against this. There are results showing a high heritability of bone density with age [58-62] while other studies show heritability is higher in premenopausal compared to postmenopausal women [63]. This suggests that there are distinct genetic components to BMD heritability - one influencing the accrual of peak bone mass and the other influencing the postmenopausal rate of bone loss [63, 64].

Heritability -Hip geometry

An important factor contributing to bone strength in addition to BMD, is bone size and geometry which also have reasonably strong genetic component and may help to explain the importance of family history as a risk factor for hip fracture. The heritability for hip axis length (HAL) for

example, is estimated at 62% [65, 66]. The increased risk of hip fracture due to longer HAL is independent of BMD and body height and may provide in part an explanation for racial differences in hip fracture incidence. Asian and African women have 40-60% lower risk for hip fracture compared to Caucasian and also a significantly shorter HAL which seems to account for a substantial part of the risk reduction [67].

Heritability – Quantitative ultrasound

The ultrasound properties of bone reflect both bone density and bone microarchitecture, such that the term “bone quality” is often used as an explanation of what quantitative ultrasound (QUS) measures. QUS, of the calcaneus can identify individuals with increased risk for fracture in both hip and spine independently of BMD [68, 69]. Consistently, it appears that there are a number of genes influencing ultrasound parameters rather than BMD alongside the earlier identified genes contributing to both entities [70]. QUS parameters speed of sound (SOS) and broadband ultrasound attenuation (BUA) have a heritability accounting for 61% and 45% respectively as suggested in a twin study [65]. Interestingly, it has also been shown in a mother-daughter study that some heritable effects on calcaneal microarchitecture do not appear until after menopause [71].

Heritability - Bone turnover indices

Markers of bone metabolism also have a genetic component and a high heritability for markers of bone metabolism, calcium homeostasis and the hormones regulating them has been identified in a large British twin study [10]. In this cohort the heritability was 14-80% for bone formation and resorption markers, including bone-specific alkaline phosphatase (67-80%), osteocalcin (14-44%), deoxypyridinoline (52-64%) and calcium excretion (41-61%).

The heritability for hormones was: serum-PTH (54-65%), serum-25OHD (28-57%), serum-1,25(OH)²D (53-74%) and serum concentration of vitamin D binding protein (56-66%).

Heritability – Fracture

The most important clinical trait of interest in osteoporosis is fracture, but the genetic contribution to fracture is more complex than for the other described phenotypes. In twin and family studies of wrist fracture, heritability is estimated at 25%-54% and independent of BMD [62, 72-74]. The heritability of hip fracture is higher in younger age groups, decreases with age and appears to become almost negligible (<3%) in the very elderly, over the age of 80 years [72, 75]. This reflects the fact that in older individuals, environmental factors and other factors contributing to fall risk become more relevant to fracture than genetic factors.

Among the other osteoporosis related phenotypes that have been identified as having a heritable component, but are not covered in detail here include age at menarche [62], age at menopause [72] (which is of great importance for the onset of type I (postmenopausal) osteoporosis), lean mass and muscle strength [73].

Methods in genetic research

There are several different methods commonly used to dissect the genetic contribution to complex diseases such as osteoporosis. These include linkage studies, animal studies, candidate gene studies and the most recent, genome wide association studies (GWAS). All of the methods look for evidence of association between phenotypic traits and genetic markers. Genetic markers may be single nucleotide polymorphisms (SNP's), deletion/insertion polymorphisms (DIP's) or variable number tandem repeats, (VNTR's). All of these analytical methods have both strengths and weakness in terms of their suitability for studying osteoporosis.

Linkage can be defined as the tendency of two or more genetic markers at specific positions (loci) to be inherited together as a result of their physical proximity. The closer they are to one another, the more frequently they will be inherited together. The probability that a marker locus is linked to a disease locus is expressed by the LOD-score. A LOD-score, over 3.6 is considered the criteria for evidence of linkage [74] and means that the odds are a thousand to one in favour of genetic linkage. Linkage studies are normally performed in large numbers of families and if there is linkage,

affected individuals carry the risk allele more frequently than unaffected individuals. This method is not ideally suited to the study of osteoporosis because its late onset makes it difficult to collect large families of affected members. Linkage analysis is better suited to the analysis of rare Mendelian bone diseases e.g. osteogenesis imperfecta and classic linkage studies have identified several genes regulating BMD. Polymorphisms in these genes have subsequently been found to be associated with bone phenotypes in the general population [76].

Animal studies have several advantages over human studies for the study of complex diseases. These include the possibility for standardized environmental conditions and controlled breeding resulting in a more homogeneous genome and large numbers of individuals. All animal genomes are to major extent homologous with human making it possible to identify human osteoporosis candidate genes by mapping animal models. The most notable gene to be identified in this way is the *ALOX15* gene [77].

Candidate gene association studies in populations have been the most extensively used method in research dissecting out the genetic contribution to osteoporosis. Such studies require that the gene is chosen based on its known role in the regulation of the phenotype or pathogenesis of the disease. Consequently, genes encoding cytokines and growth factors regulating bone turnover e.g. insulin growth factor 1 (*IGF-1*) and interleukin-6 (*IL-6*) have been dominant in the literature [78-81]. Other examples of studied genes include calciotropic hormones and their receptors e.g. parathyroid hormone, the vitamin D receptor and the calcium sensing receptor [82, 83]. Sex hormones, bone matrix components and most recent proteins of inflammation are likewise candidate genes studied in association with osteoporosis. A disadvantage of this approach is the often different results obtained for the same gene but in different populations. This may be explained by different mechanisms being important in different populations; different gene variants being studied and insufficient sample size. In recent years, meta-analyses of the most commonly studied osteoporosis genes have been performed [84-87]. These increase sample size and hence statistical power, therefore giving a more accurate estimate of the contribution of a gene variant to the phenotype.

Category	Candidate gene	Reference
Growth factors and cytokines	IGF-1, Insulin like growth factor IL-6, Interleukin-6	[78-81]
Calcitropic hormones and receptors	<i>PTH</i> , parathyroid hormone <i>VDR</i> , Vitamin D receptor <i>CaSR</i> , Calcium sensing receptor	[82, 83, 88]
Sex hormones and receptors	<i>ESRα</i> , Estrogen receptor α <i>AR</i> , Androgen receptor	[89, 90]
Bone matrix components	<i>COL1A1</i> , Collagen type 1α1 <i>BGP</i> , Osteocalcin	[84, 85, 91]
Inflammation	<i>ALOX 12</i> , Arachidonate 12-lipoxygenase <i>RANK</i> , Receptor activator of nuclear factor-κB <i>RANKL</i> , RANK ligand <i>OPG</i> , Osteoprotegerin	[92, 93]

Table 1. Candidate genes studied in association with bone traits

Our study is a candidate gene association study. We selected the *PTH* complex genes including the genes coding for *PTH*, *PTH1R* and *PTH2R* where *PTH1R* and *PTH2R* previously not have been investigated in relation to osteoporosis.

In *genome-wide association studies* (GWAS) the entire genome is screened with large numbers (>100,000) of single nucleotide polymorphisms (SNPs). The advantage of this method is that it is hypothesis free, and can therefore identify genetic variants in novel genes or pathways not previously known to be involved in the phenotype being investigated. A disadvantage is the high threshold for statistical significance required ($P < 5 \times 10^{-8}$), which

means that some SNPs may be overlooked that actually contribute to the phenotype but have a small effect.

Recently, several genome wide association studies (GWAS) have been performed and successfully identified SNPs contributing to the genetic regulation of BMD as well as fracture [86, 87, 94-96]. Examples of candidate genes discovered in genome wide studies are tissue specific alkaline phosphatase, cathepsin K, methylenetetrahydrofolate reductase, tumor necrosis factor receptor 2 and leptin receptor [74, 97]. Interestingly, *PTH* gene polymorphisms have recently been associated to femoral neck BMD in a GWAS [98].

***PTH* gene pathway polymorphism**

The human gene encoding for *PTH* is located at chromosome 11, for *PTH1LH* at chromosome 12, *PTH1R* at chromosome 3 and *PTH2R* at chromosome 2. There are mainly 2 single nucleotide polymorphisms (SNP's) in the *PTH* gene earlier studied in relation to bone traits, the *BstBI* and the *DraII* polymorphisms (named after their restriction enzymes). Both the *BstBI* and *Dra II* polymorphisms has been associated with BMD in adolescent Japanese girls [99] and *BstBI* alone in Japanese postmenopausal women [100] and in Japanese primary hyperparathyroidism patients [101]. On the other hand no association is seen between *BstBI* and BMD in two Chinese studies [102, 103]. There is also evidence for ethnic differences in prevalence of the *BstBI* polymorphisms between Chinese and Caucasians [104]. The (AAAG)_n repeat in the P3 promoter of *PTHR1* gene in relation to BMD has been studied in Chinese population with no association to BMD [105].

Techniques for assessment of bone mass

Several techniques are available for assessing bone mass. For diagnostic use in patients the method should be as safe and precise as possible. In addition to this, advantages such as low cost, comfortable and quick examination, good reference data and results easy to interpret are important factors. In animal research, for example, other factors are of importance. In the clinic there are however mainly two methods used, dual-energy X-ray absorptiometry (DXA) and quantitative ultrasound (QUS), which are more extensively described below. Quantitative computed tomography (QCT) is mainly used in research and there are also descriptions in the literature of magnetic resonance imaging (MRI) and digital radiogrammetry used for assessing bone mass.

Dual-energy X-ray absorptiometry

The currently used DXA technique is a development from earlier methods. The single and dual photon absorptiometry with higher doses of radiation and longer exposure time were followed by the single X-ray absorptiometry. The modern DXA technique was introduced in the 1980s and is since 1994 the standard method in assessing BMD for the purpose of diagnosing osteoporosis. DXA measurement involves two x-ray beams with different energy levels where the low-energy beam attenuates soft tissue and the high-energy beam attenuates soft tissue and bone [106]. Subtracting the low-energy scan value from the high-energy results in bone mineral content (BMC, g) and an estimate of density with areal BMD in g/cm^2 which is the entity most commonly used.

From a total body DXA scan, BMC, BMD, lean mass and fat mass can be obtained but data from regions such as hip and lumbar spine are primarily used in clinical settings. These sites are the most accurate for predicting hip or vertebral fracture respectively [6].

DXA involves exposing the patient to ionizing radiation but the radiation dose from one scan is less than one tenth of a chest x-ray (20 μSv) and lower than the average daily background radiation (est. 7 μSv) [107]. Limitations with DXA technique are inclusion of artifacts such as osteophytes, surgical implants and vertebral fractures which falsely elevates the BMD. Bone size, which is an important factor in bone strength, can

only be estimated in one plane and the (areal) BMD is in fact dependent on bone size assuming that the skeletal part measured always is as thick as it is wide. Trabecular microstructure and discrimination between cortical and trabecular bone can not be evaluated due to the relatively low resolution. The shortcomings in the DXA technique concerning bone size and structure are addressed with the quantitative computed tomography.

Quantitative computed tomography

QCT, using standard CT equipment is a technique where a three dimensional assessment of BMD is possible. Cortical and trabecular bone can be assessed separately, bone geometry can be measured and imaging of the trabecular bone structure is possible [108]. There are machines for both axial and peripheral techniques where the latter foremost is used in research. The limitations of QCT are a 10-20 fold higher radiation dose compared to DXA, limited reference data, higher cost and a need for higher skilled staff in both the lab and when interpreting the results.

Quantitative ultrasound

QUS, is a technique for estimating bone strength or quality through analyzing the alternation of an ultrasonic wave after passing trough both trabecular and cortical bone [109, 110]. The most commonly used skeletal site for this method is the heel, calcaneus. The variables speed of sound, SOS (m/s), and broadband ultrasound attenuation, BUA (db/MHz), are used and a stiffness index, SI, is derived from SOS and BUA in the Lunar Achilles® machines. The equipment is comparatively inexpensive, small and portable [111]. QUS does not predict fracture as well as a standard axial DXA but is comparable with peripheral DXA [111] with moderate to strong correlation [112]. This correlation is also earlier investigated in and applies to the Malmö OPRA cohort [113]. QUS has furthermore been shown to predict fracture independent of bone mass [114-117], hence it is believed to measure some other aspect of bone strength alongside with BMD. The ultrasound signal is influenced by several different material properties including bone mass, microarchitecture and tissue elasticity [112, 118, 119]. An obvious advantage with QUS is that the patient is not exposed to any ionizing radiation but since precision is lower than DXA the

method is not recommended for treatment follow-up or clinical trials hence not preferred for clinical assessment.

Bone geometry and biomechanical properties

Bone strength is dependent on many factors where BMD measured by DXA is the most important factor in predicting fracture [6]. The biomechanical property and geometry of a bone is however an important factor in bone strength and resistance to fracture.

Bone biomechanics

A bone will be deformed when it is subjected to load, to certain extent elastic, then plastic until the failure point, fracture. The elastic deformation is reversible, energy from the deforming force is stored in the bone until relieved and the bone will regain its original shape. If the load exceeds the elastic deformation capacity of the bone the yield point will be reached and plastic deformation with micro fractures will occur. Even higher load will finally result in complete fracture of the bone and is referred to as failure point which represents the bone strength [120].

Load can be divided into stress and strain where stress is force per unit area and strain relative deformation. The stiffness of the material represents the relationship between stress and strain before the yield point is reached and also referred to as Young's modulus.

Bones ability to withstand load from different directions is also dependent on the orientation of both osteons of the cortical bone and trabecula of the trabecular bone. Bone strength is lesser when load is applied transverse to osteons or trabecula compared to axial with these structures.

Direction of load can be divided into compressive, tensile and shear force although the in vivo situation normally comprises a combination of the three. Axial load will generate compressive load and geometrical bone strength will in this situation depend on the cross-sectional area. If bone is subjected to stretch force the load is tensile and if the force is rotational the load is shear in direction. In the typical bending situation there will be compressive

force on one side of the bone and tensile on the other. In a tubular bone diaphysis the strength then will depend on spatial distribution of material in relation to the neutral axis. The further away from the neutral axis the cortex is situated the greater is the bones resistance to both bending and shear forces.

Hip strength analysis

For evaluation of bone geometry in the hip, software from Lunar® Instruments (Madison, WI, USA) referred to as hip strength analysis, HSA, is available. A number of hip geometry and strength variables can be extracted from a DXA image of the proximal femur.

The identification of the narrowest cross section of the femoral neck (femoral neck width (mm)) is the central part of the hip strength analysis software and this cross section level is then analyzed for distribution of BMD for the subsequent calculations of the following described variables.

Cross-section moment of inertia, CSMI (cm^4), describes the bones resistance to bending forces independently of the material properties of the bone tissue and is a key parameter for describing bone strength. CSMI of the femoral neck calculated by DXA has been highly correlated to both direct measurement on cadaver [121] and high resolution QCT [122]. The formula for CSMI is $(\pi/4)(R_o^4 - R_i^4)$ where R_o is the outer and R_i the inner radius of the bone. Section modulus, SM (cm^3), also describes the resistance to bending forces and is calculated as $\text{CSMI}/\text{outer radius}$ $((\pi/4)(R_o^4 - R_i^4))/R_o$. The formula for polar moment of inertia is $(\pi/2)(R_o^4 - R_i^4)$ and describes the bone's resistance to torsion but is not used in this study since the femoral neck not is subjected to any significant rotational forces.

The anatomical structure of the hip leaves the femoral neck as a structure primarily vulnerable to bending forces and the distance from the pelvic rim to the outer aspect of femur along the femoral neck axis, hip axis length, HAL (mm), is earlier independent of age and BMD correlated to risk for hip fracture [123].

Aims of the study

The aim of this study was to evaluate the potential influence of *PTH* pathway gene polymorphisms on bone traits in elderly women. Within the *PTH* gene pathway we included the *PTH*, *PTH1LH*, *PTH1R* and *PTH2R* genes. By including previously studied polymorphisms and adding additional polymorphisms at each of these genes we aimed to cover as much genetic information as possible and determine their role in osteoporosis traits in Swedish women. The bone related traits included BMD, bone ultrasound, prevalence of fracture and bone geometry. Along the way new questions were raised and in this thesis we also addressed the problem with artifactly elevated BMD values due to degenerative changes on lumbar spine DXA scans. The ultimate aim of the study is to facilitate the identification of individuals at risk for fragility fractures.

Specific aims:

- I. To investigate the relationship between polymorphisms within the *PTH*, *PTH1LH*, *PTH1R* and *PTH2R* genes and bone traits including BMD and fracture in elderly Swedish women.
- II. To investigate, based on the results in study I where a correlation between *PTH* polymorphism and fracture independent of BMD was revealed, the relation between genetic polymorphism of *PTH*, hip fracture and bone geometry of the hip.
- III. To determine the impact of degenerative changes on lumbar spine DXA measurements over time and the implications for the clinical diagnosis of osteoporosis.
- IV. To further investigate the contribution of *PTH* pathway genes to bone size, this time using the vertebrae and additionally to determine if they contribute to degenerative changes at the lumbar spine in elderly women.

Subjects and methods

Study participants and assessment of data

The Osteoporosis Prospective Risk Assessment Study, OPRA, cohort comprises 1044 women who all were 75 years of age (75.2 ± 0.1 SD) at inclusion. 1604 women were invited between December 1995 and May 1999 and 65% (1044) agreed to participate. 99% of the participants were of Swedish origin and 1%, 12 women, were Caucasian immigrants from other European countries. The absolute majority of the women were self-ambulatory at baseline [124]. Of the 560 women who did not participate in the study, 139 could not take part due to poor health, 13 died shortly after invitation, 32 were not reached in spite of repeated phone calls and letters and the remaining 376 had other reasons than illness for not taking part in the complete investigation [91].

All at baseline participating women were re-invited after 5 and 10 years for a complete re-investigation including DXA scan and questionnaire. 715 of the women took part at the 5 year follow-up and 383 at 10 years.

At baseline DXA measurement of the lumbar spine was successfully obtained in 974 cases and of the hip in 951 cases. Reasons for exclusion were prior surgery in lumbar spine or hip respectively, obesity or disability preventing the participant from assuming the supine position needed for the DXA scan. Quantitative ultrasound, QUS, of the right heel was obtained in 854 cases. The missing 190 QUS examinations were due to technical failure of the instrument [91].

Blood samples for analysis and genotyping were obtained from 1008 of the women.

At baseline and follow-up all participants also answered a questionnaire regarding general health, medication, life style and previous falls or fractures.

Self reported fracture data at baseline included all fractures sustained between 20 and 75 years of age divided into the subgroups depending on site and age. These data were then verified by comparing to data from the X-ray files of the department of radiology at the Malmö University Hospital [125]. Fracture data from the follow-up questionnaires have continuously been verified in the same manner [126].

Informed consent was obtained from all participants and the study was approved by the Ethics Committee of Lund University.

Genotyping

Whole blood was available from 1008 women and genotyping was performed in 745-1005 of the participants.

The QIA amp 96 DNA blood kit (Qiagen, Valencia, CA, USA) was used according to the manufacturer's instructions for isolating total genomic DNA from whole blood. DNA was quantified using PicoGreen. Using 2ng of DNA per reaction genotyping was performed with allelic discrimination on amplified DNA using an ABI Prism 7900HT (Applied Biosystems, Foster City, CA, USA) with Taqman technology. A 384-well reaction plate was used under standard conditions (50°C for 2 min, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C).

The single nucleotide polymorphisms (SNPs) for *PTH* were selected from ensemble to encompass the gene and its surroundings and also according to Haploview to represent the most frequently occurring haplotypes. Some of the SNPs have been included in studies from other groups in cohorts of other ethnicities. The SNP's for *PTH1LH*, *PTH1R* and *PTH2R* were selected in order to obtain the highest amount of genetic information, taking into consideration: minor allele frequency in Caucasian populations, relative position to the gene and distance between the polymorphisms, table 2.

For the *PTH* gene we studied 6 SNP's located in and around the *PTH* region as follows; rs307253 located ~4 kb downstream; rs307247 located ~100 bp downstream; rs6254 in intron 2 (also known as *BstBI*); rs1459015 which lies ~5 kb upstream, rs10500783 located 56 kb upstream and finally rs10500784 located 56.2 kb upstream, figure 2.

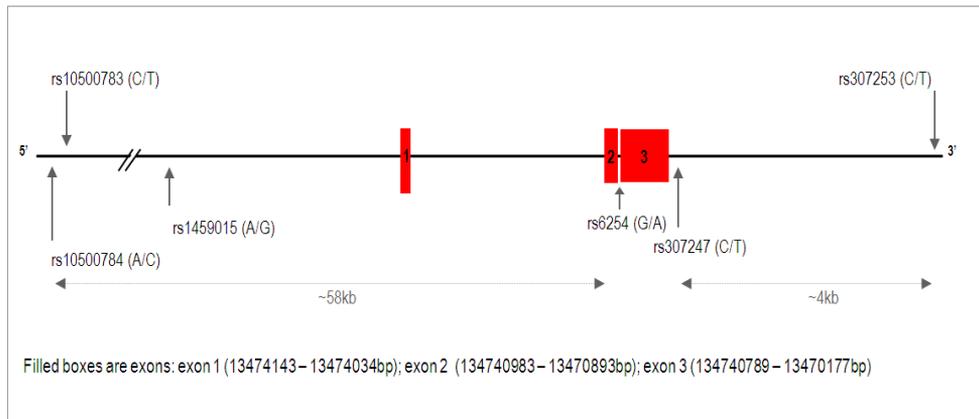


Figure 2: *PTH* gene structure and position of polymorphisms included in the study. The figure illustrates the structure of the *PTH* gene and the positions of the SNPs studied based on information from HapMap.

3 SNPs of the *PTH* gene were studied; rs805512 located in intron 3; rs10492364 in intron 4 and rs1268693 ~2 kb downstream of the gene.

In *PTH1R* we studied 3 SNPs: rs6442037 located in the first intron; rs724449 in intron 4 and rs7652849 located 3 kb downstream of the gene.

Three SNPs were also studied in *PTH2R*. These were: rs9288393 located ~2 kb upstream of the gene; rs10497900 in intron 2 and rs897083 located in intron 9.

Five common haplotypes of the *PTH* gene defined by the six SNPs in LD were identified, table 3.

SNP (base change)	Location	Homozygous (Major allele) N° (%)	Heterozygote N° (%)	Homozygous (Minor allele) N° (%)	Total
<i>PTH</i>					
rs307253 (C/T)	3'UTR	332 (44.2)	326 (43.4)	93 (12.4)	751
rs307247 (C/T)	3'UTR	332 (44.1)	327 (43.5)	93 (12.4)	752
rs6254 (G/A)	Intron 2	305 (41.0)	328 (44)	112 (15.0)	745
rs1459015 (A/G)	5'UTR	492 (65.7)	226 (30.2)	31 (4.1)	749
rs10500783 (C/T)	5' UTR	725 (72.1)	260 (25.9)	20 (2.0)	1005
rs10500784 (A/C)	5'UTR	408 (40.7)	448 (44.7)	146 (14.6)	1002
<i>PTHLH</i>					
rs805512 (T/G)	Intron 3	383 (38.4)	478 (47.9)	136 (13.6)	997
rs10492364 (G/A)	Intron 4	533 (53.1)	397 (39.5)	74 (7.4)	1004
rs1268693 (C/T)	3'UTR	295 (29.5)	523 (52.3)	182 (18.2)	1000
<i>PTH1R</i>					
rs6442037 (A/G)	Intron 3	440 (43.8)	445 (44.3)	120 (11.9)	1005
rs724449 (A/G)	Intron 4	377 (37.5)	478 (47.6)	150 (14.9)	1005
rs7652849 (C/T)	3'UTR	801 (79.7)	187 (18.6)	17 (1.7)	1005
<i>PTH2R</i>					
rs9288393 (C/G)	5'UTR	556 (55.5)	390 (38.)	55 (54.9)	1001
rs10497900 (G/T)	Intron 2	313 (31.1)	479 (47.7)	213 (21.2)	1005
rs897083 (G/A)	Intron 9	662 (65.9)	315 (31.4)	27 (2.7)	1004

Table 2. Location and allele distribution of SNP's included in the study. All SNPs were in HWE

PTH Haplotype					
SNP	HAP 5	HAP 9	HAP 2	HAP 8	HAP 1
rs10500784	C	A	A	A	A
rs10500783	C	C	T	C	C
rs1459015	A	G	A	A	A
rs6254	A	G	G	G	G
rs307247	C	T	C	T	C
rs307253	C	T	C	T	C
Haplotype frequency	36.7%	19.3%	15.3%	14.8%	13.1%

Table 3. The 5 most common haplotypes derived from the *PTH* SNPs

Bone mineral density assessment

A Lunar® DPX-L scanner (Madison, WI, USA) was used to assess BMD of the femoral neck and lumbar spine at both baseline and follow-up within the OPRA study, figure 3. These results were used in all four publications included in this thesis. Calibration of the machine using the manufacturer's phantom was performed 3 times per week and the precision coefficients were 1.6% at the femoral neck and 0.5% at the spine [127].



Figure 3. The Lunar DXA scanner where the patient is positioned with flexion of hip joints for precise lumbar scan assessment

Ultrasound measurements of the skeleton were performed using the Lunar Achilles® system. Three variables were extracted from this instrument; speed of sound (SOS), broadband ultrasound attenuation (BUA), and stiffness index (SI). The skeletal site measured was the right calcaneus except if previous fracture or injury whereas the left calcaneus was used instead. Daily calibrations ensured long-term stability of the results and the precision coefficient of the measurements was in our hands 1.5% [127].

Hip strength analysis

In publication II we used the hip strength analysis software provided by Lunar® Instruments (Madison, WI, USA). With this software, X-ray absorption data was extracted from the DXA hip image from all subjects by a single operator. Then based on the dimensions, distribution and density of bone mass in the femoral neck our variables were calculated.

The variables used in this thesis are femoral neck width (FN width, mm), hip axis length (HAL, mm), cross section moment of inertia (CSMI, cm^4) and section modulus (SM, cm^3) which are closely described in earlier chapter.

The reproducibility of this method was tested by repeating the examination five times after repositioning in the machine in six young healthy subjects. The coefficient of variance (CV) and standard error of the mean (SEM) were calculated for hip strength indices and BMD. The CV was 0.6-3.7% and SEM was 0.02-0.06.

Visual assessment of the DXA lumbar spine image

At each DXA scan site an image is produced alongside with the BMD values by the Lunar® Instruments software. All images of the lumbar spine were visually assessed, at baseline and the 5- and 10- year follow-up (age 80 and 85 years). At first the image was judged if technically good enough and then graded on the subjects of degenerative changes, fracture and scoliosis, all by one observer (MT). Disc space narrowing, vertebral osteophytes, asymmetric subchondral sclerosis or facet joint sclerosis were the criteria's for degenerative changes, figure 4. Vertebral height reduction exceeding 50% compared to adjacent vertebra and/or symmetric subchondral sclerosis were the criteria's for vertebral fracture and Cobb's angel between L1 and L4 exceeding 10 degrees for scoliosis.

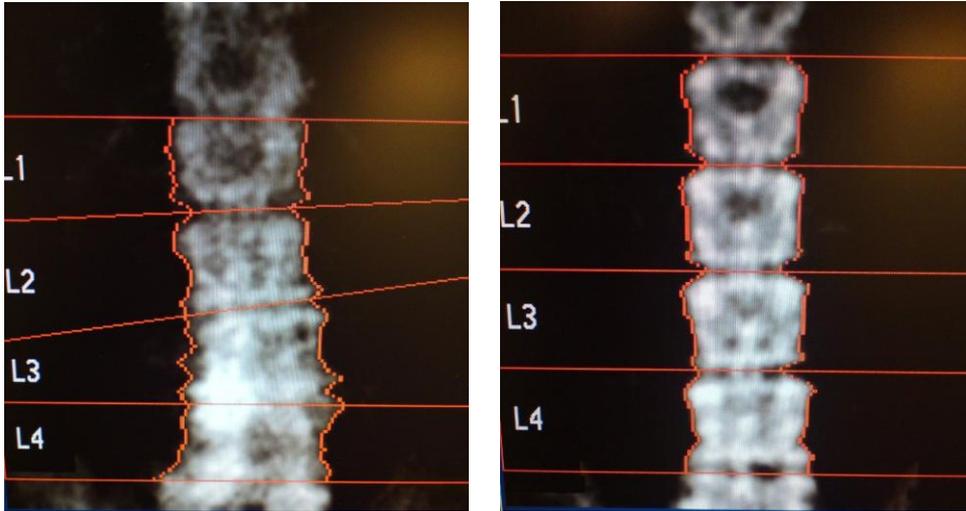


Figure 4. Examples of DXA images where the left meets criteria's for degenerative changes affecting L3 and L4. The right image is reported as normal.

For the purpose of assessing the precision of the method, every 10th of the base line scans were re-evaluated by the same observer. Every 3rd of the 10 year follow-up scans were re-evaluated by a second observer, a consultant radiologist. The Kappa values for inter- and intra-observer reproducibility on degenerative changes and scoliosis ranged from moderate to almost perfect agreement ($K= 0.43 - 0.83$). All lumbar X-rays between 2003 and 2010 among the 10 year survivor group were also evaluated on the same criteria's (82 lumbar X-rays were found among the 380 women in the 10 year follow-up group) with Kappa values in the range of moderate agreement ($K= 0.37 - 0.60$). There were not enough fractures detected for evaluation of intra- or inter-precision on that variable.

Statistical methods

SPSS versions 13.0 – 18.0 (SPSS Inc., Chicago, 2000) were used for the statistical analysis throughout this study.

Hardy-Weinberg equilibrium (HWE), to test deviation from expected allele frequencies, was performed for all polymorphisms using the χ^2 test. The distribution of alleles in a population should be constant and in equilibrium when there is no external genetic pressure from events such as mutations, non-random mating or very limited population size. HWE describes if allele frequencies differ from the expected proportions.

Linkage disequilibrium (LD) was tested between all polymorphisms pair wise using the EH and 2BY2 programs with results presented as Lewontin's D' . These results were verified and furthermore provided as the square of the correlation coefficient (r^2) by the Haploview program. Linkage disequilibrium is the occurrence of some combinations of alleles or genetic markers, such as SNP's, in a population more often or less often than would be expected from a random formation of haplotypes from alleles based on their frequencies.

The *PTH* haplotypes were determined by indirect haplotyping and defined by the six SNP's in LD using the PHASE v2.02 programme [128]. Analyses of haplotype frequencies were performed according to whether individuals carried 0, 1 or 2 copies of the haplotype allele.

Genotype and haplotype association with BMD, PTH levels, HSA and vertebral dimensions were investigated using general linear model ANOVA and univariate analysis when correcting for confounders such as BMI, s-PTH, s-calcium and smoking status. Genotypic, recessive and dominant models were used to assess the association between s-PTH and the genetic variables.

Logistic regression analysis correcting for BMI, BMD, smoking, s-PTH and s-Ca was used identifying genetic variance as predictor of fracture. These data were presented as odds ratios with 95% confidence interval (CI). In paper II, relationships between anthropometric variables and bone strength parameters were evaluated by Spearman's correlation test.

Based on a rare genotype frequency of 4% and a level of significance of $p < 0.05$ primary power analysis was calculated with regard to BMD. The study power was estimated to 80% in detecting differences of 0.063 BMD units assuming a standard deviation of 0.14 g/cm^2 .

For all the analyses, the level of significance was set at $p < 0.05$. The Bonferroni correction for multiple testing was not applied since it is considered to be too conservative for use with polymorphisms which are in linkage disequilibrium and phenotypic variables which are not independent of one another (e.g. BMD at hip and spine are correlated as is BMD and fracture)

In study I the adjusted level of significance was estimated to $p < 0.009$ equivalent to $p = 0.05$ while in papers 2 and 4 the P values reported are nominal without correction for multiple testing.

Results

Study I

The aim for study I was to investigate the association between polymorphism within the *PTH*, *PTH1R*, *PTH2R* and *PTH1LH* genes and bone traits including bone mineral density and fracture prevalence in elderly Swedish women.

All genotype frequencies within the four genes were in Hardy Weinberg equilibrium and there was a significant LD between the six SNP's of the *PTH* gene.

Genotype associations with BMD at hip and lumbar spine

Association analysis between BMD (lumbar spine and femoral neck) and the individual SNP's of the four genes found no evidence of a significant association between the individual genotypes and BMD, nor between haplotypes of the *PTH* gene and BMD.

Genotype associations with fracture and s-PTH

Fracture data were analyzed in separate categories; any fracture type occurring before baseline; any fracture type occurring between 50-75 years; any fragility fracture (hip, vertebral, proximal humerus and wrist fractures) occurring before baseline and finally, fractures occurring from 50 years onwards including prospective fractures up to 82 years.

When analysed with the individual SNP's of the *PTH* pathway genes, we found no association between the *PTH1R*, *PTH2R* and *PTH1LH* genes and any category of fracture. SNP rs307253 of the *PTH* gene was however significantly associated with both fracture between 50-75 years and when prospective fractures were added (50-82 years). Carriers of the rare allele of

this SNP were protected against fracture with overall p-values of 0.03 and 0.003 respectively.

When analysing the five common haplotypes there was a trend for carriers of haplotype 9 to have a lower frequency of fracture (p=0.018, 0.034, NS and 0.012 in respective fracture group) and carriers of haplotypes 5 (p=NS, 0.013, NS, 0.011) or haplotype 1 (p=NS, 0.02, NS, NS) to have a higher frequency of fracture. The observed associations with fracture were observed even after correction for bone density, and are therefore independent of BMD.

PTH serum level was also associated with both the individual SNP's of all genes in the PTH pathway and the haplotypes of the *PTH* gene. An association between s-PTH and the SNP's rs307247 and rs307253 was discovered, both with p=0.020 in overall significance. There was not a clear increase in s-PTH levels across the genotypes however - the highest s-PTH levels were in the heterozygotes in both cases. This could be explained in a recessive model where individuals carrying 1 or 2 copies of the variant allele had lower s-PTH levels than those carrying 2 copies of the common allele. In this model, corrected for s-calcium and s-vitamin D, p-values increased to 0.025 and 0.023 respectively. No correlation was seen between the other SNP's or haplotypes and s-PTH.

Study II

The aim of this study was to investigate the relationship between genetic polymorphisms of *PTH*, bone structure of the hip and hip fracture. The findings we obtained in study I led to the hypothesis that *PTH* polymorphism and its relationship to fracture risk, which was independent of BMD, could be through an effect on bone structure. The same genetic markers for the *PTH* gene were used as in study I.

Correlations between anthropometric variables weight and height and hip strength variables were positive and highly significant, although weight more strongly correlated to FN width, cross-section moment of inertia (CSMI) and section modulus (SM) than height.

Of the 750 genotyped women, 117 suffered a hip fracture retro- and prospectively. All fractures were results of low energy trauma and sustained

after the age of 45. Excluding the three women whose fractures occurred before the age of 55 did not alter the results. Current height and weight did not differ between the groups but when comparing reported height at 20 years of age, the women who suffered from hip fracture were taller ($p=0.001$).

The strength index section modulus and femoral neck BMD were lower and hip axis length greater in the hip fracture group as expected. There was however no significant differences between femoral neck width and cross sectional moment of inertia between the groups. All quantitative ultrasound variables were lower in the hip fracture group.

Genotype associations with femoral neck strength parameters and quantitative ultrasound

Carriers of haplotype 9, which appeared to be protective against fracture as reported in paper I, were significantly associated with lower FN width and CSMI ($p=0.029$ and 0.049 respectively). There was also a trend towards lower SM ($p=0.063$) but no correlation with HAL after correction for current length and height. When we compared individuals who carried one or two copies of haplotype 9 against those who did not carry haplotype 9, the level of significance increased to $p=0.008$, 0.018 and 0.029 for FN width, CSMI and SM respectively. Haplotype 5 and haplotype 1, known to be a risk factor for fracture in paper 1, were not associated with any parameters of the hip strength analysis. One of the individual *PTH* polymorphisms, rs1459015, was associated with FN width ($p=0.046$). All association analysis of the haplotypes or individual SNP's of the *PTH* gene in relation to quantitative ultrasound parameters of bone were negative.

Carriers of haplotype 9 were also protected against hip fracture ($p=0.043$) in analogy with fracture overall in paper 1. No other genotypes were associated with hip fracture, including haplotype 5 and 1 which previously we found to be risk factors for fracture overall in the OPRA cohort.

Study III

Since we could not confirm an expected correlation between *PTH* pathway polymorphism and vertebral BMD in study I we hypothesized that lumbar degenerative changes may have distorted the BMD values. The aim of study III was to determine the impact of degenerative changes on lumbar spine dual energy x-ray absorptiometry (DXA) measurements over time and the implications for the clinical diagnosis of osteoporosis.

Degenerative changes were detected on the DXA scan in 5% - 36% of the vertebrae at baseline, the higher prevalence being further distal in the lumbar spine. At the 10 year follow-up (age 85 years) there was a massive increase in the prevalence of degenerative changes to 20% - 72%. The same trend was observed in lumbar scoliosis with a prevalence of 10.5% at baseline, increasing to 15.8% at 5 years and 26.1% at 10 years follow-up. Presence of vertebral fractures was only judged to be present in up to 2.9% of the cases at baseline and less at follow-up.

The BMD of the individual lumbar vertebrae followed the same pattern as degenerative changes with a significant gradient towards higher BMD further distally. This gradient persisted at both 5 and 10 years. There was also a significant increase in BMD over time both when analysing all OPRA participants and the sub-group of 383 women who attended all three visits. If excluding all women with degenerative changes at any vertebra BMD appeared stable over time but the gradient with increasing BMD in proximal-distal direction remained from L1 to L3. The participants of the non-degenerative subgroup also had lower BMD at all vertebrae at both baseline and follow-up compared to the entire study population.

If translating the BMD values into prevalence of osteoporosis (T-score < 2.5 SD) at baseline and follow-up there is a decrease in the proportion with osteoporosis measured in the lumbar spine over time in elderly women. This decrease is replaced with a stable development over time if excluding all women with degenerative changes or a small increase if evaluating T-score of the most proximal vertebrae in this subgroup. It is also noticeable that if using the combined level L2-L4, as earlier recommended in the literature, a considerable smaller number of women is diagnosed with osteoporosis compared to if using L1-L2. At 75 years of age the percentages are 33% and 46% respectively and the difference persists over

time. At the femoral neck, where manifestations such as degenerative changes probably are less common, there is a clear increase in women diagnosed with osteoporosis over time, 31%, 44% and 48% at baseline, 5 and 10 year follow-up respectively. Degenerative changes or osteoarthritis of the hip mainly engages the femoral head and not the neck.

Study IV

The aim for study IV was to investigate, based on the results in study II and III, whether *PTH* pathway genes play a role in determining bone dimensions in the vertebrae or conversely if these genes contribute to degenerative changes at the lumbar spine in elderly women. The same genetic markers within the genes *PTH*, *PTHLH*, *PTHR1* and *PTHR2* were used as in study I and II.

Relationship between PTH pathway SNPs and vertebral size

The average vertebral width of vertebrae L1-L4 at baseline was significantly lower in women who were homozygous for the common allele of *PTH* polymorphisms rs6254 ($p=0.038$), rs10500784 ($p=0.025$). Women who did not carry haplotype 5 also had lower vertebral width ($p=0.037$). Vertebral width decreased in a step-wise fashion in women with 0, 1 and 2 copies of haplotype 9.

The *PTH2R* polymorphism rs9288393 was significantly associated with vertebral width ($p=0.001$), although it wasn't a stepwise relationship across the genotypes. The heterozygous genotype displayed the highest average vertebral width. No correlations with the other *PTH* pathway genes and vertebral size were seen. The results were essentially unchanged whether women using bisphosphonates at baseline ($n=32$) were included or excluded from the analyses.

Body height and vertebral dimensions are strongly correlated (0.11-0.46) and the results reported above are not corrected for these. Correction for body height and weight eliminated all significant values.

Relationship between PTH pathway SNPs, scoliosis and vertebral deformity

The *PTH2R* gene was significantly associated with lumbar scoliosis. Women who carried two copies of the rare allele of the *PTH2R* polymorphism rs10497900 had significantly higher prevalence of scoliosis than did other genotypes (Pearson χ^2 p= 0.003).

The *PTH2R* gene was also significantly associated with spine degenerative changes. *PTH2R* SNP rs897083 was significantly associated (Pearson χ^2 p=0.001) with degenerative changes at any vertebra at baseline. Carriers of the rare allele were over represented among women with degenerative changes in the lumbar spine, incremental with number of alleles carried.

Individuals who were homozygous for the common allele of the *PTH* SNP rs1459015 showed a non-significant trend towards higher prevalence of lumbar scoliosis at baseline. Carriers of one or two copies of haplotype 9 demonstrated a trend towards lower prevalence of scoliosis.

Discussion

In this thesis we have investigated if genetic polymorphisms within the PTH pathway are associated with bone traits related to osteoporosis in a cohort of elderly Swedish women. We have also reported on the prevalence and distribution of degenerative changes in the lumbar spine and its impact on bone densitometry in the same cohort.

Study design and methods

The OPRA study was designed to identify risk factors for fracture in elderly women. The study design also recognized that osteoporosis and osteoporosis-related fractures were linked to multiple factors and to the interaction between potential risk factors; lifestyle, health and medications, falls, muscle strength and balance, bone density, bone metabolism and genes.

The comprehensive evaluation of four genes within the PTH complex in relation to bone phenotypes has to our knowledge not been carried out previously. The essential role of PTH in calcium homeostasis and the powerful anabolic effect of PTH on the skeleton when used as a drug were in addition to this our main reasons for the study. In the literature there are both positive [99, 104, 129] and negative [102, 130] associations between polymorphisms of the *PTH* and *PTH1R* genes and BMD have been reported. There are also indications that ethnic [104], age and gender [131] differences might influence the impact on bone traits from PTH genes, which makes our genetically homogenous cohort of females suitable for the purpose of eliminating these confounding factors. The age of the women at inclusion is furthermore appropriate for the studied traits and the possibility for longitudinal evaluation of BMD, prospective fracture data and degenerative changes over 10 years, was invaluable.

PTH polymorphism and bone mineral density

The unexpected negative findings on association between *PTH* pathway polymorphism and BMD could have several explanations. Earlier studies of these genes have been performed mainly in Chinese and Japanese populations and ethnic differences can explain differences in the results obtained, from a genetic perspective. Environmental exposures are also likely to be different and could have an important effect on calcium homeostasis. The most commonly studied SNP, *BstBI* (rs6254), is included in our study but the difference in the results observed compared to other cohorts could be affected by additional differences in other factors including age, gender, cohort size, methods and sample size. Another plausible explanation for difficulties replicating associations between *PTH* gene polymorphisms and bone phenotypes could be that genetic variation affecting PTH function might be lethal since the tight regulation of calcium ion homeostasis and maintenance of serum calcium levels is vital to organ function.

PTH polymorphism and bone geometric properties

The results in study I, although negating our main hypothesis of an effect on bone mineral density, suggested a possible correlation between *PTH* haplotypes and prevalence of fracture independent of BMD.

There is evidence that PTH exerts direct effect on bone other than modulation of density such as differential effect on trabecular and cortical bone [132], this is also seen when PTH is used as treatment for osteoporosis [133, 134]. In paper II we showed that the common haplotype 9 of the *PTH* gene was associated with the hip strength parameters FN width, CSMI and SM. These parameters represent both skeletal size and relation between trabecular and cortical bone, confirming *PTH* polymorphism as a genetic factor for bone strength independent of BMD.

These results did not explain the associations seen in study I whereas it indicated decreased femoral neck strength among haplotype 9 carriers. However, the haplotype did not correlate with hip fracture specifically but with fracture overall. The only hip strength parameter directly associated with hip fracture was hip axis length as has also been observed by other

investigators [123]. A possible reason for the inconclusive results indicates that our cohort might still have been too small and in particular the number of hip fractures too low suggesting that the hypothesis should be tested in a larger population.

Densitometry and lumbar spine artifacts

DXA of the lumbar spine and femoral neck are key assessments in patient investigations of osteoporosis. It is widely recognized that there are inherent problems with the interpretation of spinal DXA measurements, nevertheless spinal DXA is the primary or main secondary outcome of pharmaceutical trial for new drugs to treat osteoporosis. The rationale for trying to use also the image information from the DXA scans was based on the fact that the images together with the measurement data are the foundation for diagnosing osteoporosis in the clinic.

The results of study III can primarily be criticized for the subjective method of visually detecting artifacts on the DXA image. There are however indications that it is in fact the visually detectable degenerative manifestations that significantly alter the DXA results and not the non-detectable artifacts such as minor degeneration, compression fractures or aortic calcifications [135]. If for example a vertebral fracture is misinterpreted as a degenerative manifestation it does not alter the consequence, in our method, that vertebra is excluded from the DXA result. Furthermore, even if precision is moderate our outcome indicates a considerable gain in identifying individuals with lumbar osteoporosis. There is also the obvious risk of misinterpretation of drug-effect using the standard report of either L2-L4 or L1-L4, a risk that could be reduced if one takes the visually detectable artifacts into consideration and applies L1- L2 or even only L1 for diagnosis in standard reports particularly in the elderly.

The results in study III furthermore point towards that the lumbar BMD values we used in study I were falsely elevated and since PTH treatment has a massive anabolic effect on the trabecular bone in the vertebrae [136, 137] the presumed relation between *PTH* polymorphism and lumbar BMD should be re-evaluated.

PTH polymorphism, vertebral dimensions and degenerative changes

There were significant associations between several *PTH* and *PTH2R* polymorphisms and vertebral width. This result may be mediated through an effect on skeletal size since significance was lost after correcting for height and weight. Since we did not see any association between *PTH* pathway genetic markers, height and weight our results suggests that load bearing is more important for bone size. The response to load bearing might be mediated through a PTH regulated mechanism [138].

The association between *PTH2R* polymorphism rs897083 and visible vertebral degenerative changes is still to be explained. There are however earlier studies where a locus including the *PTH2R* gene is linked with familial early-onset osteoarthritis [54, 55]. Since degenerative changes in the spine to a certain extent are caused by osteoarthritis in the facet joints we can conclude that our observed association has a biologically plausible basis.

Strengths of the study

An obvious strength with this study is our large, homogenous and well characterized cohort of over one thousand 75 years old Swedish women. These characteristics make it especially suitable for studies on genetics since there are well known age, gender and ethnic factors contributing to genetic diversity. The broad coverage of the genes involved in the PTH pathway investigated in relation to bone phenotypes is furthermore to our knowledge unique. The study on visual assessment of the DXA images is moreover the first of its kind, including the assessments of each individual vertebra and with potentially important clinical implications although the method is not fully quantitative.

Limitations

Our homogenous cohort could be considered a limitation given that the results may not be applicable to other ages, ethnicities or men. Further studies confirming our results in both elderly women and other populations are necessary. Even though our cohort size can be regarded as large the case frequencies of some variables such as prevalence of hip and vertebral fractures still were too low for powerful statistics. The use of multiple

genetic markers and a number of phenotypes aiming for a broad coverage of the hypothesis is also a limitation due to increased risk for mass significance and random findings. Although the method of detecting degenerative changes on DXA images clearly indicated that there is a gradient with increasing changes distally, visual assessment of the DXA scan does not provide a true diagnosis of the observations. Regrettably, the OPRA study did not include standard spinal radiograms of the thoracic or lumbar spine, hence it does not allow for systematic comparison. Neither is lateral or morphometric DXA included. Possibly, a lateral scan could have improved identification of vertebral fractures; however, the main purpose of the study was not to ascertain causality but to see if it was possible to enhance the utility of routine spinal DXA investigations.

Discussion summary

Polymorphism within the *PTH* gene complex has in this study been associated to fracture risk, bone strength, degenerative manifestations and s-PTH. The mechanisms behind these observations are still to be precisely described but it is a strong indication that *PTH* gene pathway variation is of importance for bone phenotypes and fracture risk. Investigation of bone structure with pQCT in relation to *PTH* gene polymorphism would be highly interesting since our results suggests that bone geometry, composition and architecture are variable which are influenced. Genetic variation within the *PTH2R* gene and its relation to degenerative manifestations and familial osteoarthritis points towards inflammation mediated effects which is a field currently studied in relation to osteoporosis.

Clinical implications

There are obvious clinical implications for the results of study III. When evaluating lumbar DXA scans of the elderly woman, the chance of detecting osteoporosis is up to double if one compares the old standard L2-L4 (28%) with L1-L2 (56%) among the 85 year old women. It is furthermore, according to our results, very important to assess the prevalence of degenerative manifestations when interpreting effects of any drug designed to increase bone mass, since contrary to observations in postmenopausal women, spinal BMD rather appear to increase over time with advancing age.

The outcome of the genetic studies indicates it might be possible to add certain genes to risk algorithms to improve identification of patients at risk, although it needs to be borne in mind that each gene contributes only with a small additional risk in complex diseases. Moreover, osteoporosis and osteoporosis-related fractures risk is multifactorial, hence risk assessment must include multiple parameters.

Conclusions and future prospects

In conclusion our results suggest an association between *PTH* haplotypes and fracture risk independent of bone mass in elderly Swedish women. The mechanism behind this relationship is however not clear and further investigation is merited since PTH is the main regulator of calcium homeostasis and bone metabolism.

Furthermore we have evaluated association between *PTH* haplotypes, bone dimensions and structure in the hip and can conclude that there is a positive relationship with bone size at the hip although this does not directly explain hip fracture incidence, which has other important non-genetic risk factors.

Moreover we can conclude that there is a very high prevalence of lumbar degenerative changes in elderly women which can be detected through visual assessment of the DXA image. These degenerative changes have a significant impact on BMD and should be considered when interpreting the results of a lumbar DXA scan.

Finally we can also conclude that polymorphisms in the genes of the PTH system do not make a significant contribution to vertebral dimensions, at least in elderly women. However, variation in the *PTH2R* gene may contribute to the age associated degenerative manifestations that develop at the spine.

The immediate future project is to continue the visual evaluation of lumbar DXA scans to capture also earlier changes; in a cohort of middle aged perimenopausal women and in a cohort of young adult 25 year old women. The objectives will be assessment of prevalent lumbar degenerative changes and scoliosis aiming to further investigate and understand the natural course of these manifestations in relation to spinal DXA. There is also blood for genotyping available in these cohorts allowing future genetic association studies.

Acknowledgements

This study was performed at the Department of Orthopedics in Malmö and there are many co-workers in the research out-patient clinic and our research group who have contributed to this book in different ways. I wish to express my sincere gratitude to all of them. There are however some people who made the extra effort I am particularly grateful towards:

All the 1044 women who voluntarily participated in this study.

Kristina Åkesson, my tutor and supervisor, for enthusiastic and professional guidance through the difficulties of science and not giving up on her sometimes frustrated student.

Fiona McGuigan, my co-tutor, for endless patience in explaining genetics to an orthopedic surgeon, improving my language skills and introducing me to Irish whisky.

Holger Luthman, my co-tutor, for scientific advice and many joyful discussions about genetics, rats and other important things in life.

Paul Gerdhem, who together with the staff in the research out-patient department made an enormous effort in collecting all data used in this study.

Lisa Jansson, who meticulously made all the genotyping lab work.

Jack Besjakov, for invaluable help and advice in the radiological field.

Henrik Ahlberg, who contributed with his knowledge in bone geometry and biomechanics.

Karl Obrant, for scientific advice and always being encouraging.

Jan Åke Nilsson, for statistical advice.

Dana, my wise and beautiful wife, for encouragement, love and never-ending patience with a boring husband behind the computer.

Stella, my lovely daughter, who's smile makes it all worthwhile!

Sammanfattning på Svenska

Om osteoporos hos äldre kvinnor

Skelettegenskaper, benfraktur och paratyroideahormon systemets gener

Bakgrund

Osteoporos och dess konsekvens, ca 70000 frakturer per år i Sverige, är en av de stora folksjukdomarna. Detta innebär ett stort lidande för vår åldrande befolkning och en stor kostnad för samhället.

Paratyroideahormon, PTH, är ett hormon som insöndras från bisköldkörtlarna och via receptorer löser ut kalcium från skelettet vid kalkbrist. PTH används även som läkemedel och fungerar då märkligt nog uppbyggande på skelettet när det ges som daglig injektion.

Livsstilsfaktorer såsom viktnedgång, rökning och inomhusvistelse har negativ effekt på bentätheten men osteoporos beräknas ändå till cirka 70% bero på genetiska faktorer.

Definitionen på osteoporos är en bentäthet på under -2,5 standard deviation, SD, jämfört unga vuxna mätt med Dual energy X-ray absorptiometry, DXA. Denna teknik har dock sina brister och mätresultaten påverkas bland annat av degenerativa förändringar i skelettet.

Målsättning

Osteoporos är en mycket långsam process och för att på ett bättre sätt kunna förebygga frakturer behöver vi så tidigt som möjligt identifiera de individer som i framtiden kommer att utveckla sjukdomen.

I denna avhandling har vi fokuserat på två infallsvinklar inom detta problem. 1) Att identifiera individer med genetiska varianter inom PTH gen systemet som medför ökad risk för osteoporos och fraktur. 2) Att identifiera individer med degenerativa förändringar i skelettet som kan påverka resultatet vid en bentäthetsmätning och försena osteoporos-diagnosen.

Material och Metod

Vi har använt mätresultat, blodprover och frågeformulär från 1044 kvinnor inom Malmö Osteoporosis Prospective Risk Assessment studien, OPRA. Dessa kvinnor var 75 år gamla vid inklusion och till 99% av svenskt ursprung. Man har sedan kallat samma individer för uppföljande bentäthetsmätning efter 5 och 10 år samt registrerat alla nytillkomna frakturer.

Inom arbete 1, 2 och 4 har vi använt blodproverna för analys av variation inom PTH gen systemet. Detta med avseende på förekomst av polymorfismer (genetiska varianter) som därefter enskilt associerats till bentäthet, frakturförekomst, skelettdimensioner och degenerativa förändringar. Vi har också undersökt vanligt förekommande kombinationer av polymorfismer, så kallade haplotyper, och deras relation till ovanstående benvariabler.

Inom arbete 3 har vi visuellt bedömt alla DXA bilderna på ländryggen från inklusion och uppföljning med avseende på störande förändringar i skelettet.

Resultat

Slutsats i delarbete 1 är att polymorfism inom PTH genen är associerad till förekomst av fraktur inom vår kohort men oberoende av bentäthet. Association med serum koncentration av PTH förelåg också.

Slutsats i delarbete 2 är att det även finns ett samband mellan PTH polymorfism och bendimensioner i vår kohort men den förklarar inte sambandet i arbete 1. Längden på lårbenshalsen är den mest signifikanta prediktorn inom skelett dimension för höft fraktur i OPRA populationen.

Slutsats i delarbete 3 är att degenerativa och andra förändringar i ländryggen, som visuellt kan detekteras på DXA bilden, är en betydande orsak till falskt förhöjda resultat vid bentäthets mätning hos äldre kvinnor. Genom att utesluta kotor som är drabbade och/eller att alltid välja de två övre ländkotorna kan man minska detta problem.

Slutsats i delarbete 4 är att det verkar finnas ett samband även mellan PTH polymorphism och kotdimensioner dock sannolikt medierat via generell benstorlek. Vi ser också ett samband mellan polymorphism inom PTH receptor 2 genen, skolios och degenerativa förändringar i ländryggen. Dessa oväntade fynd behöver verifieras i andra populationer.

Sammanfattningsvis indikerar våra studier att variation inom PTH-gen systemet har betydelse för frakturnrisk, skelettgeometri och benstruktur. Vidare, visar vi att visuell bedömning av DXA mätningar kan bidra till förbättrad diagnostik av osteoporos hos äldre kvinnor.

References

1. Cooper, C., *Epidemiology of osteoporosis*. Osteoporos Int, 1999. **9 Suppl 2**: p. S2-8.
2. Raisz, L.G., *Pathogenesis of osteoporosis: concepts, conflicts, and prospects*. J Clin Invest, 2005. **115**(12): p. 3318-25.
3. *Consensus development conference: prophylaxis and treatment of osteoporosis*. Osteoporos Int, 1991. **1**(2): p. 114-7.
4. Kanis, J.A., *Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: synopsis of a WHO report. WHO Study Group*. Osteoporos Int, 1994. **4**(6): p. 368-81.
5. Phillips, P.J. and G. Phillipov, *Bone mineral density - frequently asked questions*. Aust Fam Physician, 2006. **35**(5): p. 341-4.
6. Marshall, D., O. Johnell, and H. Wedel, *Meta-analysis of how well measures of bone mineral density predict occurrence of osteoporotic fractures*. BMJ, 1996. **312**(7041): p. 1254-9.
7. Riggs, B.L., et al., *Changes in bone mineral density of the proximal femur and spine with aging. Differences between the postmenopausal and senile osteoporosis syndromes*. J Clin Invest, 1982. **70**(4): p. 716-23.
8. Dempster, D.W., et al., *Preserved three-dimensional cancellous bone structure in mild primary hyperparathyroidism*. Bone, 2007. **41**(1): p. 19-24.
9. Rubin, M.R., et al., *The natural history of primary hyperparathyroidism with or without parathyroid surgery after 15 years*. J Clin Endocrinol Metab, 2008. **93**(9): p. 3462-70.
10. Hunter, D., et al., *Genetic contribution to bone metabolism, calcium excretion, and vitamin D and parathyroid hormone regulation*. J Bone Miner Res, 2001. **16**(2): p. 371-8.
11. Arnaud, C.D. and S.D. Sanchez, *The role of calcium in osteoporosis*. Annu Rev Nutr, 1990. **10**: p. 397-414.
12. Rupperecht, M., et al., *Bone microarchitecture of the calcaneus and its changes in aging: a histomorphometric analysis of 60 human specimens*. J Orthop Res, 2006. **24**(4): p. 664-74.
13. Seibel, M.J., *Biochemical markers of bone turnover part II: clinical applications in the management of osteoporosis*. Clin Biochem Rev, 2006. **27**(3): p. 123-38.

14. Seibel, M.J., *Biochemical markers of bone remodeling*. Endocrinol Metab Clin North Am, 2003. **32**(1): p. 83-113, vi-vii.
15. Garnero, P. and P.D. Delmas, *Biochemical markers of bone turnover. Applications for osteoporosis*. Endocrinol Metab Clin North Am, 1998. **27**(2): p. 303-23.
16. van der Klift, M., C.D. de Laet, and H.A. Pols, *Assessment of fracture risk: who should be treated for osteoporosis?* Best Pract Res Clin Rheumatol, 2005. **19**(6): p. 937-50.
17. Kanis, J.A., *Diagnosis of osteoporosis and assessment of fracture risk*. Lancet, 2002. **359**(9321): p. 1929-36.
18. Henry, M.J., et al., *Fracture risk score and absolute risk of fracture*. Radiology. **259**(2): p. 495-501.
19. Kanis, J.A., et al., *FRAX and the assessment of fracture probability in men and women from the UK*. Osteoporos Int, 2008. **19**(4): p. 385-97.
20. Chrischilles, E., T. Shireman, and R. Wallace, *Costs and health effects of osteoporotic fractures*. Bone, 1994. **15**(4): p. 377-86.
21. Sernbo, I. and O. Johnell, *Consequences of a hip fracture: a prospective study over 1 year*. Osteoporos Int, 1993. **3**(3): p. 148-53.
22. Melton, L.J., 3rd, et al., *Perspective. How many women have osteoporosis?* J Bone Miner Res, 1992. **7**(9): p. 1005-10.
23. Eastell, R., et al., *Classification of vertebral fractures*. J Bone Miner Res, 1991. **6**(3): p. 207-15.
24. Cooper, C., et al., *Incidence of clinically diagnosed vertebral fractures: a population-based study in Rochester, Minnesota, 1985-1989*. J Bone Miner Res, 1992. **7**(2): p. 221-7.
25. van Staa, T.P., et al., *Epidemiology of fractures in England and Wales*. Bone, 2001. **29**(6): p. 517-22.
26. Kanis, J.A., et al., *Long-term risk of osteoporotic fracture in Malmo*. Osteoporos Int, 2000. **11**(8): p. 669-74.
27. Cummings, S.R. and L.J. Melton, *Epidemiology and outcomes of osteoporotic fractures*. Lancet, 2002. **359**(9319): p. 1761-7.
28. Gabriel, S.E., et al., *Direct medical costs attributable to osteoporotic fractures*. Osteoporos Int, 2002. **13**(4): p. 323-30.
29. Compston, J., *Action Plan for the prevention of osteoporotic fractures in the European Community*. Osteoporos Int, 2004. **15**(4): p. 259-62.
30. Cumming, R.G., et al., *Calcium intake and fracture risk: results from the study of osteoporotic fractures*. Am J Epidemiol, 1997. **145**(10): p. 926-34.
31. Tang, B.M., et al., *Use of calcium or calcium in combination with vitamin D supplementation to prevent fractures and bone loss in people aged 50 years and older: a meta-analysis*. Lancet, 2007. **370**(9588): p. 657-66.

32. Bischoff-Ferrari, H.A., et al., *Fracture prevention with vitamin D supplementation: a meta-analysis of randomized controlled trials*. JAMA, 2005. **293**(18): p. 2257-64.
33. Boonen, S., et al., *Addressing the musculoskeletal components of fracture risk with calcium and vitamin D: a review of the evidence*. Calcif Tissue Int, 2006. **78**(5): p. 257-70.
34. Cranney, A., et al., *Meta-analyses of therapies for postmenopausal osteoporosis. IX: Summary of meta-analyses of therapies for postmenopausal osteoporosis*. Endocr Rev, 2002. **23**(4): p. 570-8.
35. Wells, G.A., et al., *Alendronate for the primary and secondary prevention of osteoporotic fractures in postmenopausal women*. Cochrane Database Syst Rev, 2008(1): p. CD001155.
36. Rizzoli, R., et al., *Subtrochanteric fractures after long-term treatment with bisphosphonates: a European Society on Clinical and Economic Aspects of Osteoporosis and Osteoarthritis, and International Osteoporosis Foundation Working Group Report*. Osteoporos Int. **22**(2): p. 373-90.
37. Reginster, J.Y., et al., *Effects of long-term strontium ranelate treatment on the risk of nonvertebral and vertebral fractures in postmenopausal osteoporosis: Results of a five-year, randomized, placebo-controlled trial*. Arthritis Rheum, 2008. **58**(6): p. 1687-95.
38. Neer, R.M., et al., *Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis*. N Engl J Med, 2001. **344**(19): p. 1434-41.
39. Greenspan, S.L., et al., *Effect of recombinant human parathyroid hormone (1-84) on vertebral fracture and bone mineral density in postmenopausal women with osteoporosis: a randomized trial*. Ann Intern Med, 2007. **146**(5): p. 326-39.
40. Cummings, S.R., et al., *Denosumab for prevention of fractures in postmenopausal women with osteoporosis*. N Engl J Med, 2009. **361**(8): p. 756-65.
41. Kanis, J.A., et al., *Smoking and fracture risk: a meta-analysis*. Osteoporos Int, 2005. **16**(2): p. 155-62.
42. Kanis, J.A., et al., *Alcohol intake as a risk factor for fracture*. Osteoporos Int, 2005. **16**(7): p. 737-42.
43. Linden, C., et al., *A school curriculum-based exercise program increases bone mineral accrual and bone size in prepubertal girls: two-year data from the pediatric osteoporosis prevention (POP) study*. J Bone Miner Res, 2006. **21**(6): p. 829-35.
44. Joakimsen, R.M., J.H. Magnus, and V. Fonnebo, *Physical activity and predisposition for hip fractures: a review*. Osteoporos Int, 1997. **7**(6): p. 503-13.

45. Gensure, R.C., T.J. Gardella, and H. Juppner, *Parathyroid hormone and parathyroid hormone-related peptide, and their receptors*. *Biochem Biophys Res Commun*, 2005. **328**(3): p. 666-78.
46. Brown, E.M., *Physiology and pathophysiology of the extracellular calcium-sensing receptor*. *Am J Med*, 1999. **106**(2): p. 238-53.
47. Kawashima, H., S. Torikai, and K. Kurokawa, *Localization of 25-hydroxyvitamin D3 1 alpha-hydroxylase and 24-hydroxylase along the rat nephron*. *Proc Natl Acad Sci U S A*, 1981. **78**(2): p. 1199-203.
48. Brommage, R., et al., *Daily treatment with human recombinant parathyroid hormone-(1-34), LY333334, for 1 year increases bone mass in ovariectomized monkeys*. *J Clin Endocrinol Metab*, 1999. **84**(10): p. 3757-63.
49. Nemere, I. and D. Larsson, *Does PTH have a direct effect on intestine?* *J Cell Biochem*, 2002. **86**(1): p. 29-34.
50. Brown, A.J., et al., *The roles of calcium and 1,25-dihydroxyvitamin D3 in the regulation of vitamin D receptor expression by rat parathyroid glands*. *Endocrinology*, 1995. **136**(4): p. 1419-25.
51. Kronenberg, H.M., *Developmental regulation of the growth plate*. *Nature*, 2003. **423**(6937): p. 332-6.
52. Stewart, A.F., et al., *Biochemical evaluation of patients with cancer-associated hypercalcemia: evidence for humoral and nonhumoral groups*. *N Engl J Med*, 1980. **303**(24): p. 1377-83.
53. Usdin, T.B., C. Gruber, and T.I. Bonner, *Identification and functional expression of a receptor selectively recognizing parathyroid hormone, the PTH2 receptor*. *J Biol Chem*, 1995. **270**(26): p. 15455-8.
54. Meulenbelt, I., et al., *Strong linkage on 2q33.3 to familial early-onset generalized osteoarthritis and a consideration of two positional candidate genes*. *Eur J Hum Genet*, 2006. **14**(12): p. 1280-7.
55. Min, J.L., et al., *Mutation analysis of candidate genes within the 2q33.3 linkage area for familial early-onset generalised osteoarthritis*. *Eur J Hum Genet*, 2007. **15**(7): p. 791-9.
56. Gueguen, R., et al., *Segregation analysis and variance components analysis of bone mineral density in healthy families*. *J Bone Miner Res*, 1995. **10**(12): p. 2017-22.
57. Videman, T., et al., *Heritability of BMD of femoral neck and lumbar spine: a multivariate twin study of Finnish men*. *J Bone Miner Res*, 2007. **22**(9): p. 1455-62.
58. Christian, J.C., et al., *Heritability of bone mass: a longitudinal study in aging male twins*. *Am J Hum Genet*, 1989. **44**(3): p. 429-33.
59. Smith, D.M., et al., *Genetic factors in determining bone mass*. *J Clin Invest*, 1973. **52**(11): p. 2800-8.
60. Pocock, N.A., et al., *Genetic determinants of bone mass in adults. A twin study*. *J Clin Invest*, 1987. **80**(3): p. 706-10.

61. Krall, E.A. and B. Dawson-Hughes, *Heritable and life-style determinants of bone mineral density*. J Bone Miner Res, 1993. **8**(1): p. 1-9.
62. Cummings, S.R., et al., *Risk factors for hip fracture in white women*. Study of Osteoporotic Fractures Research Group. N Engl J Med, 1995. **332**(12): p. 767-73.
63. Seeman, E., et al., *Reduced bone mass in daughters of women with osteoporosis*. N Engl J Med, 1989. **320**(9): p. 554-8.
64. Lutz, J. and R. Tesar, *Mother-daughter pairs: spinal and femoral bone densities and dietary intakes*. Am J Clin Nutr, 1990. **52**(5): p. 872-7.
65. Arden, N.K., et al., *The heritability of bone mineral density, ultrasound of the calcaneus and hip axis length: a study of postmenopausal twins*. J Bone Miner Res, 1996. **11**(4): p. 530-4.
66. Slemenda, C.W., et al., *The genetics of proximal femur geometry, distribution of bone mass and bone mineral density*. Osteoporos Int, 1996. **6**(2): p. 178-82.
67. Cummings, S.R., et al., *Racial differences in hip axis lengths might explain racial differences in rates of hip fracture*. Study of Osteoporotic Fractures Research Group. Osteoporos Int, 1994. **4**(4): p. 226-9.
68. Hans, D., et al., *Ultrasonographic heel measurements to predict hip fracture in elderly women: the EPIDOS prospective study*. Lancet, 1996. **348**(9026): p. 511-4.
69. Ross, P., et al., *Predicting vertebral deformity using bone densitometry at various skeletal sites and calcaneus ultrasound*. Bone, 1995. **16**(3): p. 325-32.
70. Howard, G.M., et al., *Genetic and environmental contributions to the association between quantitative ultrasound and bone mineral density measurements: a twin study*. J Bone Miner Res, 1998. **13**(8): p. 1318-27.
71. Danielson, M.E., et al., *Familial resemblance of bone mineral density (BMD) and calcaneal ultrasound attenuation: the BMD in mothers and daughters study*. J Bone Miner Res, 1999. **14**(1): p. 102-10.
72. Snieder, H., A.J. MacGregor, and T.D. Spector, *Genes control the cessation of a woman's reproductive life: a twin study of hysterectomy and age at menopause*. J Clin Endocrinol Metab, 1998. **83**(6): p. 1875-80.
73. Nguyen, T.V., et al., *Bone mass, lean mass, and fat mass: same genes or same environments?* Am J Epidemiol, 1998. **147**(1): p. 3-16.
74. Huang, Q.Y. and A.W. Kung, *Genetics of osteoporosis*. Mol Genet Metab, 2006. **88**(4): p. 295-306.
75. Michaelsson, K., et al., *Genetic liability to fractures in the elderly*. Arch Intern Med, 2005. **165**(16): p. 1825-30.
76. Ralston, S.H. and B. de Crombrughe, *Genetic regulation of bone mass and susceptibility to osteoporosis*. Genes Dev, 2006. **20**(18): p. 2492-506.
77. Klein, R.F., et al., *Regulation of bone mass in mice by the lipoxxygenase gene Alox15*. Science, 2004. **303**(5655): p. 229-32.

78. Rivadeneira, F., et al., *Estrogen receptor beta (ESR2) polymorphisms in interaction with estrogen receptor alpha (ESR1) and insulin-like growth factor I (IGF1) variants influence the risk of fracture in postmenopausal women.* J Bone Miner Res, 2006. **21**(9): p. 1443-56.
79. Nordstrom, A., et al., *Interleukin-6 promoter polymorphism is associated with bone quality assessed by calcaneus ultrasound and previous fractures in a cohort of 75-year-old women.* Osteoporos Int, 2004. **15**(10): p. 820-6.
80. Kim, J.G., et al., *Relationship of osteocalcin and matrix Gla protein gene polymorphisms to serum osteocalcin levels and bone mineral density in postmenopausal Korean women.* Menopause, 2006. **13**(3): p. 467-73.
81. Wang, Y.B., et al., *The human calcium-sensing receptor and interleukin-6 genes are associated with bone mineral density in Chinese.* Yi Chuan Xue Bao, 2006. **33**(10): p. 870-80.
82. Uitterlinden, A.G., et al., *The association between common vitamin D receptor gene variations and osteoporosis: a participant-level meta-analysis.* Ann Intern Med, 2006. **145**(4): p. 255-64.
83. Vezzoli, G., et al., *R990G polymorphism of calcium-sensing receptor does produce a gain-of-function and predispose to primary hypercalciuria.* Kidney Int, 2007. **71**(11): p. 1155-62.
84. Mann, V. and S.H. Ralston, *Meta-analysis of COL1A1 Sp1 polymorphism in relation to bone mineral density and osteoporotic fracture.* Bone, 2003. **32**(6): p. 711-7.
85. Mann, V., et al., *A COL1A1 Sp1 binding site polymorphism predisposes to osteoporotic fracture by affecting bone density and quality.* J Clin Invest, 2001. **107**(7): p. 899-907.
86. Rivadeneira, F., et al., *Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies.* Nat Genet, 2009. **41**(11): p. 1199-206.
87. Stykarsdottir, U., et al., *New sequence variants associated with bone mineral density.* Nat Genet, 2009. **41**(1): p. 15-7.
88. Laaksonen, M.M., et al., *Associations of vitamin D receptor, calcium-sensing receptor and parathyroid hormone gene polymorphisms with calcium homeostasis and peripheral bone density in adult Finns.* J Nutrigenet Nutrigenomics, 2009. **2**(2): p. 55-63.
89. Ioannidis, J.P., et al., *Differential genetic effects of ESR1 gene polymorphisms on osteoporosis outcomes.* JAMA, 2004. **292**(17): p. 2105-14.
90. Tofteng, C.L., et al., *Polymorphisms in the CYP19 and AR genes--relation to bone mass and longitudinal bone changes in postmenopausal women with or without hormone replacement therapy: The Danish Osteoporosis Prevention Study.* Calcif Tissue Int, 2004. **74**(1): p. 25-34.
91. Gerdhem, P., et al., *Association of the collagen type 1 (COL1A 1) Sp1 binding site polymorphism to femoral neck bone mineral density and wrist*

- fracture in 1044 elderly Swedish women. Calcif Tissue Int, 2004. 74(3): p. 264-9.*
92. Ichikawa, S., et al., *Human ALOX12, but not ALOX15, is associated with BMD in white men and women. J Bone Miner Res, 2006. 21(4): p. 556-64.*
 93. Kim, J.G., et al., *Association between osteoprotegerin (OPG), receptor activator of nuclear factor-kappaB (RANK), and RANK ligand (RANKL) gene polymorphisms and circulating OPG, soluble RANKL levels, and bone mineral density in Korean postmenopausal women. Menopause, 2007. 14(5): p. 913-8.*
 94. Richards, J.B., et al., *Collaborative meta-analysis: associations of 150 candidate genes with osteoporosis and osteoporotic fracture. Ann Intern Med, 2009. 151(8): p. 528-37.*
 95. Styrkarsdottir, U., et al., *Multiple genetic loci for bone mineral density and fractures. N Engl J Med, 2008. 358(22): p. 2355-65.*
 96. Richards, J.B., et al., *Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. Lancet, 2008. 371(9623): p. 1505-12.*
 97. Cheung, C.L., et al., *Confirmation of linkage to chromosome 1q for spine bone mineral density in southern Chinese. Hum Genet, 2006. 120(3): p. 354-9.*
 98. Guo, Y., et al., *IL21R and PTH may underlie variation of femoral neck bone mineral density as revealed by a genome-wide association study. J Bone Miner Res. 25(5): p. 1042-8.*
 99. Katsumata, K., et al., *Association of gene polymorphisms and bone density in Japanese girls. J Bone Miner Metab, 2002. 20(3): p. 164-9.*
 100. Hosoi, T., et al., *Association study of parathyroid hormone gene polymorphism and bone mineral density in Japanese postmenopausal women. Calcif Tissue Int, 1999. 64(3): p. 205-8.*
 101. Kanzawa, M., et al., *Parathyroid hormone gene polymorphisms in primary hyperparathyroidism. Clin Endocrinol (Oxf), 1999. 50(5): p. 583-8.*
 102. Deng, H.W., et al., *Tests of linkage and/or association of genes for vitamin D receptor, osteocalcin, and parathyroid hormone with bone mineral density. J Bone Miner Res, 2002. 17(4): p. 678-86.*
 103. Zhou, X.G., et al., *Parathyroid hormone gene with bone phenotypes in Chinese. Biochem Biophys Res Commun, 2003. 307(3): p. 666-71.*
 104. Dvornyk, V., et al., *Differentiation of Caucasians and Chinese at bone mass candidate genes: implication for ethnic difference of bone mass. Ann Hum Genet, 2003. 67(Pt 3): p. 216-27.*
 105. Lei, S.F., et al., *The (GT)_n polymorphism and haplotype of the COL1A2 gene, but not the (AAAG)_n polymorphism of the PTHR1 gene, are associated with bone mineral density in Chinese. Hum Genet, 2005. 116(3): p. 200-7.*

106. Blake, G.M. and I. Fogelman, *Technical principles of dual energy x-ray absorptiometry*. Semin Nucl Med, 1997. **27**(3): p. 210-28.
107. Njeh, C.F., et al., *Radiation exposure in bone mineral density assessment*. Appl Radiat Isot, 1999. **50**(1): p. 215-36.
108. Ito, M., *Recent progress in bone imaging for osteoporosis research*. J Bone Miner Metab. **29**(2): p. 131-40.
109. Gluer, C.C., *Quantitative ultrasound techniques for the assessment of osteoporosis: expert agreement on current status. The International Quantitative Ultrasound Consensus Group*. J Bone Miner Res, 1997. **12**(8): p. 1280-8.
110. Barkmann, R., et al., *A method for the estimation of femoral bone mineral density from variables of ultrasound transmission through the human femur*. Bone, 2007. **40**(1): p. 37-44.
111. Njeh, C.F., C.M. Boivin, and C.M. Langton, *The role of ultrasound in the assessment of osteoporosis: a review*. Osteoporos Int, 1997. **7**(1): p. 7-22.
112. Njeh, C.F., et al., *Is quantitative ultrasound dependent on bone structure? A reflection*. Osteoporos Int, 2001. **12**(1): p. 1-15.
113. Gerdhem, P., et al., *Ultrasound of the phalanges is not related to a previous fracture. A comparison between ultrasound of the phalanges, calcaneus, and DXA of the spine and hip in 75-year-old women*. J Clin Densitom, 2002. **5**(2): p. 159-66.
114. Gluer, C.C., et al., *Association of five quantitative ultrasound devices and bone densitometry with osteoporotic vertebral fractures in a population-based sample: the OPUS Study*. J Bone Miner Res, 2004. **19**(5): p. 782-93.
115. Karlsson, M.K., et al., *Age, gender, and fragility fractures are associated with differences in quantitative ultrasound independent of bone mineral density*. Bone, 2001. **28**(1): p. 118-22.
116. Huopio, J., et al., *Calcaneal ultrasound predicts early postmenopausal fractures as well as axial BMD. A prospective study of 422 women*. Osteoporos Int, 2004. **15**(3): p. 190-5.
117. Stewart, A., V. Kumar, and D.M. Reid, *Long-term fracture prediction by DXA and QUS: a 10-year prospective study*. J Bone Miner Res, 2006. **21**(3): p. 413-8.
118. Hans, D., et al., *Do ultrasound measurements on the os calcis reflect more the bone microarchitecture than the bone mass?: a two-dimensional histomorphometric study*. Bone, 1995. **16**(3): p. 295-300.
119. Hans, D., T. Fuerst, and F. Duboeuf, *Quantitative ultrasound bone measurement*. Eur Radiol, 1997. **7**(10): p. 43-50.
120. Turner, C.H. and D.B. Burr, *Basic biomechanical measurements of bone: a tutorial*. Bone, 1993. **14**(4): p. 595-608.
121. Yoshikawa, T., et al., *Geometric structure of the femoral neck measured using dual-energy x-ray absorptiometry*. J Bone Miner Res, 1994. **9**(7): p. 1053-64.

122. Ramamurthi, K., et al., *An in vivo comparison of hip structure analysis (HSA) with measurements obtained by QCT*. Osteoporos Int.
123. Leslie, W.D., et al., *Prediction of hip and other osteoporotic fractures from hip geometry in a large clinical cohort*. Osteoporos Int, 2009. **20**(10): p. 1767-74.
124. Gerdhem, P., et al., *Association between 25-hydroxy vitamin D levels, physical activity, muscle strength and fractures in the prospective population-based OPRA Study of Elderly Women*. Osteoporos Int, 2005. **16**(11): p. 1425-31.
125. Gerdhem, P. and K. Akesson, *Rates of fracture in participants and non-participants in the Osteoporosis Prospective Risk Assessment study*. J Bone Joint Surg Br, 2007. **89**(12): p. 1627-31.
126. Gerdhem, P., et al., *Associations between homocysteine, bone turnover, BMD, mortality, and fracture risk in elderly women*. J Bone Miner Res, 2007. **22**(1): p. 127-34.
127. Karlsson, M.K., et al., *Bone mineral density assessed by quantitative ultrasound and dual energy X-ray absorptiometry. Normative data in Malmo, Sweden*. Acta Orthop Scand, 1998. **69**(2): p. 189-93.
128. Stephens, M., N.J. Smith, and P. Donnelly, *A new statistical method for haplotype reconstruction from population data*. Am J Hum Genet, 2001. **68**(4): p. 978-89.
129. Scillitani, A., et al., *A functional polymorphism in the PTHRI promoter region is associated with adult height and BMD measured at the femoral neck in a large cohort of young caucasian women*. Hum Genet, 2006. **119**(4): p. 416-21.
130. Lei, S.F., et al., *The VDR, COL1A1, PTH, and PTHRI gene polymorphisms are not associated with bone size and height in Chinese nuclear families*. J Bone Miner Metab, 2005. **23**(6): p. 501-5.
131. Lei, S.F., et al., *Bone mineral density and five prominent candidate genes in Chinese men: associations, interaction effects and their implications*. Maturitas, 2005. **51**(2): p. 199-206.
132. Calvi, L.M., et al., *Activated parathyroid hormone/parathyroid hormone-related protein receptor in osteoblastic cells differentially affects cortical and trabecular bone*. J Clin Invest, 2001. **107**(3): p. 277-86.
133. Neer, M., et al., *Treatment of postmenopausal osteoporosis with daily parathyroid hormone plus calcitriol*. Osteoporos Int, 1993. **3 Suppl 1**: p. 204-5.
134. Reeve, J., et al., *Anabolic effect of human parathyroid hormone fragment on trabecular bone in involutional osteoporosis: a multicentre trial*. Br Med J, 1980. **280**(6228): p. 1340-4.
135. Hayirlioglu, A., et al., *The importance of severity of arthrosis for the reliability of bone mineral density measurement in women*. Rheumatol Int, 2009. **29**(4): p. 371-5.

136. Chen, P., et al., *Increases in BMD correlate with improvements in bone microarchitecture with teriparatide treatment in postmenopausal women with osteoporosis*. J Bone Miner Res, 2007. **22**(8): p. 1173-80.
137. Recker, R.R., et al., *Cancellous and cortical bone architecture and turnover at the iliac crest of postmenopausal osteoporotic women treated with parathyroid hormone 1-84*. Bone, 2009. **44**(1): p. 113-9.
138. Uchida, A., et al., *The effect of mechanical stress on cultured growth cartilage cells*. Connect Tissue Res, 1988. **17**(4): p. 305-11.

Original publications I-IV