Johanna Korvala

IN QUEST OF GENETIC SUSCEPTIBILITY TO DISORDERS MANIFESTING IN FRACTURES

ASSESSING THE SIGNIFICANCE OF GENETIC FACTORS IN FEMORAL NECK STRESS FRACTURES AND CHILDHOOD NON-OI PRIMARY OSTEOPOROSIS
JOHANNA KORVALA

IN QUEST OF GENETIC SUSCEPTIBILITY TO DISORDERS MANIFESTING IN FRACTURES
Assessing the significance of genetic factors in femoral neck stress fractures and childhood non-OI primary osteoporosis

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Abstract

Osteoporosis is a bone disorder that leads to a reduction in bone volume, deterioration of bone microarchitecture and therefore increased fracture risk. Bone disorders such as osteoporosis commonly have both genetic and environmental components. Family and twin studies have shown the importance of genetics in bone formation and health, but most of the genetic factors contributing to bone formation are still largely unknown.

The aim of this thesis was to search for and identify genetic factors that predispose to two different bone disorders manifesting in fractures, namely femoral neck stress fractures and childhood primary osteoporosis without features of OI (i.e. non-OI primary osteoporosis). Furthermore, in vitro studies were performed to elucidate the importance and mechanism of action of identified genetic factors in non-OI primary osteoporosis.

By using candidate gene analyses we identified predisposing alleles, haplotypes and their interactions that increased the risk for femoral neck stress fractures in young male military conscripts. The conscripts lacking the CTR C allele and/or VDR C-A haplotype had a three-fold increased risk for femoral neck stress fractures compared to the carriers of both. Furthermore, conscripts carrying the LRP5 A-G-G-C haplotype had a three-fold increased risk for femoral neck stress fractures and in combination with VDR C-A haplotype a four-fold increased risk for stress fractures. These associations were mediated by low body weight and BMI.

In the search for genetic factors of non-OI primary osteoporosis in children and adolescent, two novel mutations in LRP5 and two more variants in WNT3A and DKK1 were found in patients. The variants were also observed in the affected family members, but not in the control group. The effects of these variants were examined in in vitro studies and the results showed that some LRP5 mutations and the WNT3A variant might reduce bone formation by decreasing the canonical Wnt signalling activity.

Keywords: bone and bones, candidate gene analysis, femoral neck stress fractures/genetics, osteoporosis/genetics, signal transduction, Wnt proteins
Osteoporoosi on luustosairaus, joka alentaa luuntiheyttä ja heikentää luun rakennetta ja siten lisää murtumien riskiä. Osteoporoosin kaltaiset luusairaudet ovat usein monitekijäisiä tauteja, joiden syntyyn vaikuttavat sekä perinnölliset että ympäristölliset tekijät. Perhe- ja kaksosutkimukset ovat osoittaneet perinnöllisten tekijöiden olevan tärkeitä luun muodostuksessa ja terveydessä, mutta nämä tekijät ovat kuitenkin vielä suurelta osin tuntemattomia.

Tutkimustyön tavoitteena oli etsiä ja tunnistaa perinnöllisiä tekijöitä, jotka altistavat kahdelle luunmurtumina ilmenevälle sairaudelle: reisiluunkaulan rasitusmurtumille ja lasten primaarisen osteoporoosille. Lisäksi primaarisen osteoporoosille altistavien perinnöllisten tekijöiden merkitystä ja vaikutusmekanismia tutkittiin in vitro-kokeilla.


Lapsuudessa tai varhaisnuoruudessa puhkeavan primaarisen osteoporoosin perinnöllisten tekijöiden etsinnässä löydettiin kaksi uutta mutaatiota LRP5-geenistä ja yhteen kaksi uutta muutosta WNT3A- ja DKK1-geeneistä. Uusien ehdokasgeenien löydöstä uskottiin primaarisen osteoporoosin syntyyn tukea se, että muutokset löydettiin potilaiden lisäksi heidän sairailta sukulaisiltaan eikä muutoksia havaittu kontrolliaineistossa. Uusien mutaatioiden mahdollisia vaikutuksia tutkittiin in vitro-kokein, jotka osoittivat, että eräät LRP5-geenin mutaatiot ja WNT3A-geenin muutos alentavat kanonisen Wnt-signalointireittin aktiivisuutta ja voivat siten vähentää luunmuodostusta.

Asiasanat: ehdokasgeenianalyysi, kanoninen Wnt signalointireitti, luu, osteoporoosi, perinnöllisyystiede, reisiluunkaulan rasitusmurtuma
Omistettu äidilleni
"On sanoo "onko, onkohan?" ja epäily masentaa maailman. Ei sanoo: "eikö, eiköhän!" ja näemme vuorien siirtyvän."
Lauri Viita
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Setting up the in vitro studies was a task of its own, and the help from Ph.D. Minna Komu and Ph.D. Antti Railo and M.D., Ph.D. Marja-Riitta Väisänen was instrumental in getting the studies started. I wish to thank them all for their help.

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Oulu, April 2012

Johanna Korvala
Abbreviations

APC    adenomatous polyposis coli
AXIN1  axis inhibitor 1
AXIN2  axis inhibitor 2
AGE    agarose gel electrophoresis
AR     androgen receptor
β-catenin  cadherin-associated protein β
β-Gal  β-galactosidase
BMD    bone mineral density
BMP    bone morphogenetic protein
BMU    basic multicellular unit
bp     base pair
CaMK   calmodulin kinase
CaMKKβ calcium/calmodulin-dependent protein kinase beta
CaMKIV calcium/calmodulin-dependent protein kinase IV
CCLR   cell culture lysis reagent
CHO    Chinese hamster ovarian cells
CM     conditioned medium
COL    collagen
CPRG   chlorophenol red β-galactopyranoside
CREB   cAMP response element-binding protein
CSGE   conformation sensitive gel electrophoresis
CT     calcitonin
CTR    calcitonin receptor
CYP19A1 aromatase
D’     linkage disequilibrium
DKK1   dickkopf-1
DKK2   dickkopf-2
DMSO   dimethyl sulphoxide
DSH    dishevelled
DXA    dual-energy X-ray absorptiometry
EDTA   ethylenediaminetetraacetic acid
EGF    epidermal growth factor
ER/ESR estrogen receptor
FBS    fetal bovine serum
FZD    frizzled-receptor
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Term</th>
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<td>GBP</td>
<td>GSK3β binding protein</td>
</tr>
<tr>
<td>GSK3β</td>
<td>glycogen synthase kinase 3-β</td>
</tr>
<tr>
<td>GWAS</td>
<td>genome-wide association study</td>
</tr>
<tr>
<td>HBM</td>
<td>high bone mass</td>
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<tr>
<td>IJO</td>
<td>idiopathic juvenile osteoporosis</td>
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<tr>
<td>IL-6</td>
<td>interleukin-6</td>
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<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
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<tr>
<td>Lef</td>
<td>lymphoid enhancing factor</td>
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<td>LRP5/6*</td>
<td>low density lipoprotein receptor-related protein 5/6 in human</td>
</tr>
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<td>LRP5/6*</td>
<td>gene encoding for LRP5/6 in human</td>
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<td>Lrp5/6*</td>
<td>low density lipoprotein receptor-related protein 5/6 in mouse</td>
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<tr>
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<td>gene encoding for Lrp5/6 in mouse</td>
</tr>
<tr>
<td>MARK3</td>
<td>microtubule affinity-regulating kinase 3</td>
</tr>
<tr>
<td>Mesd</td>
<td>mesoderm development</td>
</tr>
<tr>
<td>MLPA</td>
<td>multiplex ligation-dependent probe amplification</td>
</tr>
<tr>
<td>MM</td>
<td>multiple myeloma</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>NT</td>
<td>nucleotide</td>
</tr>
<tr>
<td>OI</td>
<td>osteogenesis imperfecta-syndrome</td>
</tr>
<tr>
<td>OPG</td>
<td>osteoprotegerin</td>
</tr>
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<td>osteoporosis-pseudoglioma syndrome</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>pQCT</td>
<td>peripheral quantitative computed tomography</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative Real-time PCR</td>
</tr>
<tr>
<td>r²</td>
<td>correlation coefficient</td>
</tr>
<tr>
<td>RANK</td>
<td>receptor activator of nuclear factor receptor κB</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator of nuclear factor κB ligand</td>
</tr>
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<td>RLU</td>
<td>relative luciferase unit</td>
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<td>RT-PCR</td>
<td>reverse transcriptase PCR</td>
</tr>
<tr>
<td>SAP</td>
<td>shrimp alkaline phosphatase</td>
</tr>
<tr>
<td>sFRP</td>
<td>secreted frizzled related protein</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SIFT</td>
<td>sorting intolerant from tolerant - tool</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<tr>
<td>SOST</td>
<td>sclerostin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>STF</td>
<td>SuperTOPflash reporter construct</td>
</tr>
<tr>
<td>Tcf</td>
<td>T-cell factor</td>
</tr>
<tr>
<td>Th</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TPH1</td>
<td>tryptophan hydroxylase 1</td>
</tr>
<tr>
<td>TPH2</td>
<td>tryptophan hydroxylase 2</td>
</tr>
<tr>
<td>T-score</td>
<td>standard deviation in young normal subjects</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>Wif-1</td>
<td>Wnt inhibitory factor</td>
</tr>
<tr>
<td>WNT</td>
<td>wingless-related murine mammary tumour virus (MMTV) integration site family member</td>
</tr>
<tr>
<td>YWTD</td>
<td>tyrosine-tryptophan-threonine-aspartic acid</td>
</tr>
<tr>
<td>Z-score</td>
<td>standard deviation score in age-matched population</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine i.e. serotonin</td>
</tr>
<tr>
<td>5-HTT</td>
<td>5-hydroxytryptamine transporter</td>
</tr>
<tr>
<td>5-HTR1B</td>
<td>5-hydroxytryptamine receptor 1B</td>
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</tbody>
</table>

*the specified notations are applied to all proteins and genes used in the thesis.
List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


*Equal contribution
## Contents

Abstract

Tiivistelmä

Acknowledgements

Abbreviations

List of original articles

1 Introduction

2 Review of the literature

  2.1 Bone .......................................................... 25
     2.1.1 Structure ................................................. 25
     2.1.2 Composition ............................................. 28
  2.2 Bone formation: modelling vs. remodelling .................. 31
  2.3 Bone disorders ................................................ 32
     2.3.1 Fractures ................................................. 33
     2.3.2 Stress fractures ........................................... 34
     2.3.3 Osteoporosis .............................................. 35
     2.3.4 Primary osteoporosis ................................... 36
     2.3.5 Osteopetrotic conditions and HBM disorders ......... 39
  2.4 Bone and canonical Wnt signalling pathway ............... 41
  2.5 Bone and serotonin pathway .................................. 43
  2.6 Inheritance of bone characters/quality ....................... 45
     2.6.1 Genetics of osteoporosis .............................. 45
     2.6.2 Candidate genes in stress fractures ................. 46
     2.6.3 The LRP5 gene mutations ............................ 48
     2.6.4 Components of the canonical Wnt signalling pathway in bone ............................................... 50

3 Outlines of the present study

4 Material and methods

     4.1 Patient and control sets (I-III) .......................... 55
        4.1.1 Military conscripts with femoral neck stress fractures (I) .... 55
        4.1.2 Non-OI primary osteoporosis (II, III) ...................... 55
     4.2 Genetic analyses (I-III) ........................................ 56
        4.2.1 Polymerase chain reaction (PCR) (I-III) ................. 56
        4.2.2 Conformation sensitive gel electrophoresis (CSGE) (I, III) .... 57
        4.2.3 Multiplex ligation-dependent probe amplification (MLPA) (II) .................................................. 58
4.2.4 Sequencing (I-III) ................................................................. 59
4.2.5 Genotyping of 15 SNPs in the candidate genes (I) .................. 59

4.3 Functional analyses (II-III) .......................................................... 60
4.3.1 Computational analyses (II-III) .................................................. 60
4.3.2 Constructs (II-III) ................................................................. 60
4.3.3 Expression and detection of LRP5 and DKK1 (II-III) ............... 61
4.3.4 Producing Wnt3a- and DKK1-conditioned media (II-III) ....... 62
4.3.5 Luciferase gene reporter assay (II-III) ...................................... 62
4.3.6 Measuring luciferase activity (II-III) ........................................ 63
4.3.7 Quantitative-PCR (II) ............................................................. 63

4.4 Statistical methods (I-III) ............................................................. 64

5 Results ......................................................................................... 65
5.1 Genetic factors in femoral neck stress fractures (I) ....................... 65
5.1.1 Clinical findings (I) ............................................................... 65
5.1.2 Candidate gene analysis (I) .................................................... 66
5.1.3 Association analysis of SNPs (I) ............................................. 66
5.1.4 Linkage disequilibrium, haplotype and interaction analyses (I) ................................. 68

5.2 Studying the components of the canonical Wnt signalling pathway in non-OI primary osteoporosis (II-III, unpublished results) ..................................................................... 69
5.2.1 Clinical features (II-III) ....................................................... 70
5.2.2 Identification of mutations associated with non-OI primary osteoporosis (II-III) .......... 71
5.2.3 Functional significance of the variants (II-III) ....................... 76

6 Discussion .................................................................................. 83
6.1 Haplotype association in femoral neck stress fractures (I) ............ 83
6.1.1 Previous research on the genetics of stress fractures .............. 83
6.1.2 Genetic predisposition for fractures and/or reduced BMD ...... 84
6.1.3 Body weight and bone density .............................................. 86
6.1.4 Future directions ................................................................. 87

6.2 Mutations and variants associated with non-OI primary osteoporosis (II-III) .......................... 87
6.2.1 Clinical considerations ...................................................... 88
6.2.2 Location and significance of the variants ............................. 88
6.2.3 The in vitro analyses .......................................................... 90
6.2.4 Bone, Lrp5 and serotonin pathway ...................................... 91
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.2.5 Future directions</td>
<td>92</td>
</tr>
<tr>
<td>7 Conclusions and future perspectives</td>
<td>95</td>
</tr>
<tr>
<td>References</td>
<td>97</td>
</tr>
<tr>
<td>Appendix</td>
<td>125</td>
</tr>
<tr>
<td>References for the supplemental table 1</td>
<td>135</td>
</tr>
<tr>
<td>Original articles</td>
<td>139</td>
</tr>
</tbody>
</table>
1 Introduction

Bone is a dynamic and constantly renewing tissue. Bone composition is controlled by the delicate balance between bone formation carried out by osteoblasts and bone resorption performed by osteoclasts. Disruption of this balance may result in bone disorders varying from a high bone mass phenotype to osteoporosis. Osteoporosis is the most common bone disorder and it develops as bone formation is reduced or when bone resorption is increased, which leads to porous and weak bone. In Finland, 30 000–40 000 fractures resulting from deteriorated bone are treated yearly at a cost of approximately 130 million euros as reported by the Finnish Osteoporosis Foundation. Furthermore, some 400 000 Finns have been estimated to have reduced bone density. In Europe the estimated frequency for osteoporotic fractures is 1/3 for women and 1/5 for men over 50 years of age, averaged by the International Osteoporosis Foundation.

Exploring the causes of osteoporosis and fractures is important because of the strain it places on the health care system, the economy and most importantly on personal lives. The task is challenging since osteoporosis is a multifactorial disorder with both environmental and genetic components. Several family and twin studies support the role of genetic factors in determining bone composition, bone parameters and consequently proneness to fractures. Mutations in genes contributing to bone formation or composition may hamper protein structure or function resulting in a defective bone phenotype. Indeed, mutations affecting especially rare bone disorders, such as osteogenesis imperfecta (OI), have been identified and these genes have been further studied in order to ascertain their role in the maintenance of healthy bone. Hence, knowing the genetic components of bone may contribute significantly to our understanding of the aetiology, diagnostics and treatment of bone disorders.

The aim of this thesis was to study the genetic factors affecting two different bone disorders manifesting in fractures: femoral neck stress fractures and primary osteoporosis without features of OI (i.e. non-OI primary osteoporosis). In addition, the effects of variants that associated with non-OI primary osteoporosis were examined in in vitro studies. The results of these studies add information on the genetic factors predisposing to bone fragility and fractures. Furthermore, they may benefit diagnostics of different bone disorders and promote the development of effective treatment.
2 Review of the literature

2.1 Bone

Bone is connective tissue with crucial structural and functional importance. The human body contains over 200 bones that each have intricate shapes and forms to fulfil their role and function as part of the skeletal system. The largest human bone is the femur, which can measure up to 48 cm in length (Yazar et al. 2011) while the smallest bone, the stapes, is only about 3 mm in height (Anson JB 1991, Farahani & Nooranipour 2008).

The skeletal system provides structural support, protects the internal organs and enables movement together with skin, tendons and muscles. The bone marrow within bones stores fat, which may be used as an energy source, and is the site of blood cell formation. Bone also participates in the regulation of calcium and phosphate homeostasis by releasing these into circulation from hydroxyapatite, if needed.

Bone has active metabolic and mechanical functions. The dynamic nature of bone is demonstrated by its capacity to adjust and respond to the surrounding environment, nutrients, mechanical load and changes in all these. Furthermore, bone composition and geometry is not constant throughout life but changes during different life stages: starting from woven bone at birth, human bone attains its peak mass and full strength when individuals are between twenty and thirty years of age, then composition and mass are slowly reduced later in life.

2.1.1 Structure

Bone tissue exists in two types: woven and lamellar bone. Woven bone is primitive bone tissue that is observed in embryos, newborns and during callus formation in adults (Einhorn 1996). It is characterised by undefined collagen organisation and weak structure. During skeletal growth and bone remodelling woven bone is replaced by stronger lamellar bone, which presents a regular lamellar organisation of collagens that gives bone its resistance to mechanical strain (Einhorn 1996).

Anatomically bone consists of cortical (i.e. compact) and cancellous (i.e. trabecular or spongy) bone. Cortical bone comprises about 80% of the skeleton (Arikoski et al. 2002) and is located at bone surfaces creating a firm and
protective cortex. Periosteum covers the outer surfaces of bones where they are not surrounded by articular cartilage, and serves to provide nutrients to bone and attachment site for muscles and tendons. Endosteum coats bone cavities by creating a lining of cells that actively participates in bone remodelling. Cortical bone has a tough and rigid quality that gives bones their physical endurance and makes it more persistent than cancellous bone. The functional units in cortical bone are called osteons. They are formed in lamellar bone structures surrounding Haversian canals and encompass lacunae, lamella, osteocytes and canaliculi. Lacunae are spaces in which osteocytes have been embedded during bone mineralisation, and they are connected to each other via canaliculi through which osteocytes maintain important signalling and nutritional functions. Haversian canals are vertical vascular channels that transport blood and nutrients in bone, while the perpendicular canals with the same functions are called Volkmanns’ canals. The structure of cancellous bone resembles that of a sponge or a honeycomb that is filled with bone marrow. It is usually located at long bone metaphysis and in vertebral bodies where it participates mainly in the metabolic processes in bone. (Einhorn 1996)

Aside from bone tissue, other tissue types also occur in the skeletal system. Articular cartilage is found on joint surfaces. It has shock absorbing properties, and therefore it relieves mechanical forces on bone. The medullary cavity encompasses yellow bone marrow, which consists largely of adipose tissue and acts as an energy storage and source. Red bone marrow, on the other hand, is the location of blood cell production. Finally, bones grow from their ends and in long bones the zones of dividing cells are called epiphyseal lines. (Einhorn 1996) The main structures of bone are illustrated in Figure 1.
Fig. 1. Schematic presentation of the basic structure of bone. Bone consists of cortical and cancellous bone tissue which both have specific composition. The outer surface of bone is covered with periosteum while bone cavities are filled with bone marrow and lined with endosteum. The functional units of cortical bone are called osteons. They are formed around Haversian canals and encompass alternating sections of lacunae and connecting lamellae (shown in magnification).
2.1.2 Composition

Bone is composed of cells, inorganic minerals, organic matrix, lipids and water (Boskey & Coleman 2010). The three cell types that appear in bone are osteoblasts, osteocytes and osteoclasts. In addition, bone contains bone-lining cells that originate from osteoblasts.

Osteoblasts, osteocytes and osteoclasts

Osteoblasts are mononuclear cells of mesenchymal origin. They are round cells between 20–30 µm in diameter, with a basophilic cytoplasm and a prominent Golgi apparatus and rough endoplasmic reticulum (Doty & Schofield 1976, Manolagas & Parfitt 2010). Osteoblasts are bone forming cells that deposit non-mineralised matrix in their surroundings forming the osteoid (Franz-Odendaal et al. 2006). Osteoblasts are necessary also for matrix mineralisation where they control the embedding of hydroxypatite with collagen fibres (Manolagas 2000). The average lifespan of an osteoblast is three months (Manolagas 2000), but this may vary from 1 to 200 days depending on cell fate (Manolagas & Parfitt 2010). After the secretory phase osteoblasts that are trapped within the mineralised bone matrix become osteocytes, while others may turn into bone-lining cells or die through apoptosis (Jilka et al. 2007, Manolagas 2000).

Bone-lining cells derive from osteoblasts and appear elongated and flat (Martin et al. 2008), and they have a lifespan ranging from 1 to 10 years (Manolagas & Parfitt 2010). Bone-lining cells cover quiescent bone surfaces and have been suggested to secrete collagenases that expose the underlying un-mineralised collagen matrix for osteoclastic resorption (Manolagas 2000).

Osteocytes also descend from osteoblasts. The number of osteoblasts that are transformed into osteocytes averages between 10–30% (Liu et al. 1997, Parfitt 1990). Osteocytes form the most abundant cell type in adult bone, composing 90–95% of all bone cells (Boskey & Coleman 2010). They have fusiform or spherical cell bodies with dendritic extensions (Vatsa et al. 2008), and their morphology varies depending on bone type (Rochefort et al. 2010). The human osteocyte measures approximately 10 µm × 20 µm (Mullender et al. 1996, Noble 2008) and its half-life is estimated at 25 years (Parfitt 1990). Osteocytes are connected to each other, bone-lining cells and to blood vessels by dendrites that run within small canaliculi (Palumbo et al. 1990). Previously osteocytes were considered to be inactive cells, but they are now known to form an important signalling network.
that participates in the bone remodelling response caused by mechanical loading (Bonewald 2007, Rochefort et al. 2010). According to current theory, mechanical loading changes the fluid flow within the canalicular network, which eventually results in osteocyte apoptosis. This induces homing of osteoclasts and osteoblasts to the site, which initiates bone remodelling (Rochefort et al. 2010). Furthermore, osteocytes affect osteoblast and osteoclast production and participate in regulating mineralisation together with osteoblasts (Bonewald 2007).

Osteoclasts are multinucleated bone-resorbing cells that have a haematopoietic origin (Suda et al. 1992). Osteoclast precursors are first produced in the red bone marrow where they differentiate into monocytes and later form osteoclasts by fusing together, a process induced by receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) that are produced in osteoblasts and osteocytes (Simonet et al. 1997). Osteoclasts are large cells that extend from 50 to 100 μm in diameter. They are rich with mitochondria, lysosomes and free ribosomes (Manolagas 2000) and have a lifespan of 1–25 days (Manolagas & Parfitt 2010). Osteoclasts have three functional characteristics, namely a ruffled border, a sealing zone and a secretory domain (Väänänen & Horton 1995, Väänänen et al. 2000). The ruffled border with finger-like protrusions is the resorptive organelle of osteoclasts. Adenosine triphosphate (ATP) driven proton pumps within the ruffled border membrane and intracellular vacuoles bring protons into the resorption lacuna forming an acidic environment that facilitates mineral resorption, while the excreted proteinases (e.g. lysosomal cysteine proteinases and matrix metalloproteinases) dissolve the organic matrix (Väänänen & Horton 1995, Väänänen et al. 2000). The sealing zone involves a ring of actin cytoskeleton that attaches osteoclasts tightly to the mineral matrix and isolates the excavated bone area from its surroundings to create the proper environment for bone resorption (Väänänen & Horton 1995, Väänänen et al. 2000). The dissolved matrix is endocytosed from the resorption lacuna into the osteoclast where it is transcytotically brought to the secretory domain and released on the opposite side of the bone surface into the extracellular space (Manolagas 2000, Salo et al. 1997, Väänänen & Horton 1995, Väänänen et al. 2000).

Mineral matrix

Bone is a connective tissue that is formed by collagen fibres and a mineralised matrix that give bone its key features of being tough yet elastic and resistant to
fractures. Osteoblasts deposit the extracellular matrix, but also calcium, magnesium and phosphate ions to be mineralised to hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$. Hydroxyapatite accounts for 90% of the bone mineral matrix. Not all hydroxyapatite exist in its purest form in bone, but the calcium, phosphate or hydroxide ions may be replaced by e.g. hydrogen phosphate, carbonate, magnesium, sodium or fluoride ions (Robey & Boskey 1996). The nano-crystals formed are between 1–1.5 nm × 5–25 nm × 8–40 nm (thickness/width/length) (Boskey & Coleman 2010, Burger et al. 2008, Eppell et al. 2001, Tong et al. 2003), and fitted in the spaces/holes in the collagen fibril structure (Arsenault 1988, Maitland & Arsenault 1991).

Collagen forms 95% of the organic matrix and the remaining 5% is comprised of non-collagenous proteins (Boskey & Coleman 2010). The prevailing collagen in bone is collagen I which is a trimer consisting of two $\alpha_1$ and 1 $\alpha_2$ chains (Robey & Boskey 1996). After transcription and post-translational modifications (e.g. hydroxylation, glycosylation) the three $\alpha$ chains wind together to form a collagen molecule. The helical structure that is characteristic of collagens is based on the recurrence of Gly-X-Y amino acids (X is usually proline and Y hydroxyproline) in collagen sequences that enable the formation of the triple helix (Peck et al. 1964). Finally the inter- and intramolecular cross-links that form between collagen molecules and fibrils promote the generation of collagen fibres. Other collagen types are also present in bone and these include collagens IX and XII and XIV, but also small amounts of III and V have been observed (Robey & Boskey 1996).

Osteonectin is the most abundant non-collagenous protein in bone along with osteocalcin. Osteonectin binds denatured collagen and hydroxyapatite promoting mineral deposition (Termine et al. 1981) and it also affects cell shape and matrix interactions via binding to calcium (Bassuk et al. 1993). Osteocalcin has been suggested to recruit osteoclasts and regulate mineralisation and the length of mineral crystals (Malone et al. 1982). Other proteins such as bone sialoprotein, proteoglycans and phosphoproteins take part in e.g. matrix organisation, whereas osteopontin, bone acidic glycoprotein, thrombospondin and fibronectin, that possess the amino acid sequence arginine-glycine-aspartic acid (RGD), are able to bind integrins and hence participate in attaching bone cells to the extracellular matrix (Einhorn 1996).
2.2 Bone formation: modelling vs. remodelling

Bone modelling accompanies bone formation during development and growth (Einhorn 1996). There are two forms of bone modelling through which the skeleton takes its final size and form: endochondral and intramembranous ossification. Endochondral ossification is typical in the appendicular skeleton and long bones, and here the original cartilaginous skeletal mould is replaced by woven bone and eventually by lamellar bone (Chen & Alman 2009). Intramembranous ossification entails the direct formation of bone tissue by osteoblastic cells, and it takes place in flat bones e.g. skull (Ducy & Karsenty 2000, Einhorn 1998). Bones grow in length through endochondral ossification and in width through intramembranous ossification (Einhorn 1996).

Bone remodelling occurs continuously to regenerate bone tissue in order to sustain mechanical support of the skeleton and to regulate mineral homeostasis (Eriksen 2010). Remodelling is also crucial for repairing injuries such as micro-damage or fractures (Eriksen 2010). Bone remodelling was first recognised in 1969 (Frost 1969) and thereafter the topic has been of high interest in the hope of discovering new ways of treating bone disorders.

Bone remodelling encompasses a cycle of events including attracting osteoclasts to the site of the event, resorption of old bone by osteoclasts followed by their apoptosis and secretion of new bone tissue (osteoid) by osteoblasts (Einhorn 1996, Eriksen 2010, Manolagas 2000). Bone resorption and formation act in concert under strict control to maintain normal bone mass, and the two functions are tightly coupled in the process. The structure that performs remodelling is called the basic multicellular unit (BMU) and it includes the osteoclasts, osteoblasts and osteocytes that co-operate in the resorption pit (site of bone remodelling) in the vicinity of canaliculi and vasculature (Frost 1969, Parfitt 1994). Homing of BMUs to the remodelling or microfracture site may be activated and guided by osteocyte apoptosis on site (Verborgt et al. 2000). The lifespan of a BMU ranges between 6 to 9 months (Parfitt 1994).

Bone remodelling occurs on periosteal, endosteal, Haversian canal and trabecular bone surfaces (Bullough et al. 1990, Einhorn 1996, Raisz & Kream 1983). In cancellous bone the BMUs move and operate along the trabecular bone surface, as presented in Figure 2. In cortical bone the BMU proceeds within the bone by excavating tunnels (called cutting cones) that are subsequently filled with osteoid by osteoblasts. The speed of bone remodelling is about 25 μm/day although the process is slower in cortical than in cancellous bone (Manolagas
On average, the whole skeleton is regenerated through this process within 10 years (Manolagas 2000, Parfitt 1994).

Fig. 2. Schematic presentation of bone remodelling in cancellous bone. Bone remodelling occurs in resorption pits where osteoclasts resorb old bone and osteoblasts produce new bone tissue called osteoid. Osteoclasts, osteoblasts and osteocytes form the basic multicellular unit (BMU) in which the cells co-operate to perform bone remodelling. Bone-lining cells originate from osteoblasts and cover quiescent bone surfaces.

2.3 Bone disorders

The foundation of healthy bone is created in early childhood and adolescence through normal bone growth and development. If this process is adversely affected, the outcome may be reflected in abnormal or insufficient peak bone mass attainment, which may influence adult stature and increase the risk of osteoporosis or other bone disorders later in life (Arikoski et al. 2002).

Bone defects appear in many forms. In general, osteopenia and osteoporosis that are caused by reduced bone quality or quantity manifest in bone pain and fractures, which result in impaired movement and reduced quality of life. Bone defects do not manifest in weakened bone alone, but the opposite phenotype is observed in osteopetrosis and high bone mass (HBM) disorders. The main characteristic of these is increased bone accrual. In addition, bone health may be affected by poor nutrition (deficiency of calcium, phosphate and vitamin D), insufficient high impact exercise, long-term diseases (e.g. anorexia), medication (e.g. glucocorticoids), trauma or infections (Arikoski et al. 2002, Kim et al. 2006). A selection of bone defects and disorders is described in the following chapters.
2.3.1 Fractures

Fracture is a non-union in bone that results from an acute load or impact. The development of fracture depends on bone strength and/or the energy of the impact: when bone strength and composition are normal, fractures are usually caused by acute high-energy impacts, while in skeletal conditions with weakened or osteoporotic bone, fractures may result from even minor falls or hits.

Fracture healing occurs generally via indirect ossification (Marsell & Einhorn 2011), and involves several signalling pathways, e.g. Wnt signalling (Chen & Alman 2009, Secreto et al. 2009) and the Indian hedgehog pathway (Ihh) (Murakami & Noda 2000). These are reviewed in detail in (Deschaseaux et al. 2009, Schindeler et al. 2008). Three distinct phases are recognised in bone healing namely inflammatory, repair and remodelling phases (Chen & Alman 2009, Marsell & Einhorn 2011). Soon after a fracture occurs a haematoma is formed on the injured site beginning the inflammatory phase, which entails recruiting mesenchymal stem cells (MSCs) to the site to form granulation tissue that provides a base for callus formation. During the repair phase, vasculature is provided on site and MSCs in the periosteum near the fracture site differentiate into osteoblasts and start to secrete osteoid (intramembranous ossification) (Chen & Alman 2009, Marsell & Einhorn 2011). Concurrently the MSCs in the granulation tissue differentiate into chondrocytes that produce cartilage, which bridges the fractured bone sections together. Cartilage is then mineralised and consequently replaced by woven bone, which is eventually replaced by cortical bone via remodelling (endochondral ossification) (Chen & Alman 2009, Marsell & Einhorn 2011).

Direct ossification rarely occurs naturally, but rather through contact or gap healing. Contact healing requires the close proximity and strict union of the fractured bone sections, and is applied in surgical repair of fractures (Marsell & Einhorn 2011). Cutting cones appear on osteons close to the ends of fracture sites and result in simultaneous re-establishment of bone union, vasculature and a Haversian system (Marsell & Einhorn 2011, Shapiro 1988). During gap healing the bony union and remodelling of the Haversian system take place at different times and via two consecutive remodelling cycles to obtain strong and anatomically correct bone structure (Marsell & Einhorn 2011, Shapiro 1988).
2.3.2 Stress fractures

Stress fractures can be classified into two types: insufficiency and fatigue fractures. Insufficiency fractures appear in patients with weak bones (resulting from e.g. osteoporosis) under normal mechanical loading (Pentecost et al. 1964), while fatigue fractures develop in normal bone that is subjected to repetitive loading that in time overcomes bone remodelling (Belkin 1980). In this thesis the term stress fracture is used for fatigue fractures.

Stress fractures result from an increase in intensity and/or duration of mechanical loading for which bone is unable to adapt efficiently (Belkin 1980). The specific aetiology of stress fractures is yet unknown but they have been suggested to develop from microcracks (Burr et al. 1985, Burr et al. 1990). These are small defects in bone caused by continuous physical strain (Martin 2003). They are approximately 100 μm in size, and are thought to result from disruption of the mineral crystallite and/or collagen networks (Martin 2003, Mohsin et al. 2006). They are normally repaired by focal bone remodelling, but until woven bone is replaced by stronger lamellar bone during the process, the remodelling site may be prone to stress fractures (Anderson & Greenspan 1996, Frost 1991). When a microcrack develops faster than BMUs are able to restore bone, a microcrack may grow into a macrofracture and eventually into a stress fracture (Anderson & Greenspan 1996, Frost 1991). Stress fractures manifest in focal bone pain that increases during exercise and eases at rest, but also local tenderness and swelling may occur (Stanitski et al. 1978).

Stress fractures are common in people subjected to heavy physical training such as athletes and military conscripts (Ha et al. 1991, Jones et al. 1989, Sterling et al. 1992), and the prevalence of stress fractures varies between 0.9 to 12.3% in conscripts (Armstrong et al. 2004, Brudvig et al. 1983, Sahi 1984). Stress fractures are detected in almost all bones, but the majority occur in the lower extremities (Milgrom et al. 1985, Sahi et al. 1996). In a study of Finnish military conscripts, stress fractures were distributed to tibia (56%), metatarsal bones (23%), heel (8%), femur (5%) and pubic bone (4%) (Sahi et al. 1996). Risk factors for stress fractures in military conscripts include increased intensity and duration of training (Jones et al. 2002), weight loss during military service (Armstrong et al. 2004), female gender and smoking (Jones et al. 2002, Lappe et al. 2001). Furthermore stress fracture risk is inversely proportional to age (Milgrom et al. 1994), and the incidence is increased in persons with low bone mineral density and bone strength, poor fitness and/or low level of previous
physical activity (Jones et al. 2002, Pouilles et al. 1989, Välimäki et al. 2005). Finally, military conscripts of small size have been suggested to have a higher risk for stress fractures (Beck et al. 1996, Givon et al. 2000).

Femoral neck stress fractures present the most serious type of stress fractures as they may have devastating consequences. These fractures are classified as tension or compression fractures depending on their location (Sormaala et al. 2007). Tension fractures pose a risk of dislocation which, when left untreated, may lead to osteonecrosis and disability or predispose to osteoarthritis (Pihlajamäki et al. 2006a, Sormaala et al. 2007). Fortunately femoral neck stress fractures are rather rare, as their occurrence ranges from 3.5 to 8% (Erne & Burckhardt 1980, Fullerton & Snowdy 1988, Volpin et al. 1990), and in Finnish military conscripts they have become even less frequent after the release of the army’s revised diagnostic and treatment instructions in 1986 and due to the increased awareness that ensued (Pihlajamäki et al. 2006a, Pihlajamäki et al. 2006b).

### 2.3.3 Osteoporosis

Osteoporosis is mainly a disorder of the elderly, and it is especially common in postmenopausal women. It is rarely detected in children and even then it usually occurs secondary to another metabolic or bone disorder and/or as a result of medication (Arikoski et al. 1999, Javaid & Cooper 2002, Kotaniemi et al. 1998), or nutritional or environmental effects (for review see (Arikoski et al. 2002)). In adults the risk factors for osteoporosis include family-history of osteoporosis, insufficient calcium and vitamin D intake, a low amount of physical activity, eating disorders, low weight and slender body build, smoking, excess alcohol consumption, medical conditions and/or medication, age, female gender (increasingly so after menopause) and hormonal factors (Marini & Brandi 2010).

More than two hundred million people in the world including approximately 1/3 of American and European postmenopausal women have osteoporosis (Marini & Brandi 2010), and according to estimates 30–50% of women and 13–30% of men will suffer an osteoporotic fracture in life (Johnell & Kanis 2005, Randell et al. 1995). Osteoporosis is increasingly recognised also in the developing countries (Cooper et al. 1992, Gullberg et al. 1997), and with the aging population and rising number of patients worldwide, healthcare services and the economy will be faced with the challenge of treating osteoporosis. Therefore, it is important to
recognise the risk factors in order to develop efficient methods for prevention, early-diagnostics and treatment of the disease.

Osteoporosis manifests in fragile bones caused by deterioration of bone microarchitecture (NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy 2001). A person attains his/her peak bone mass at around twenty years of age, and in the early fifties the amount of bone starts to decline slowly (Eisman 1999). Because excessive bone loss can progress asymptomatically even over a long period, osteoporosis is often detected only in the context of the first fractures (Unnanuntana et al. 2010, Vestergaard et al. 2005). The majority of these fractures occur at the hip, wrist or spine, but also at other sites (Rachner et al. 2011). Osteoporotic fractures affect the patient’s mobility and quality of life significantly, and may have serious long-term consequences.

Bone quality is commonly described by bone mineral density (BMD) measured using dual-energy X-ray absorptiometry (DXA). T- and/or Z-scores are used to define BMD as advised by the World Health Organisation (WHO). The T-score compares a subject’s BMD value to the average of a normal young person, related to the standard deviation (SD) of a young normal population. The Z-score, on the other hand, compares the subject’s BMD to that of an age-matched population, delineated by the number of SDs below the age-matched population. Because the Z-score takes also age into account, it is preferred when assessing BMD in children. BMD is considered normal when T-score is above -1.0, osteopenia is characterised by a T-score between -1.0 and -2.5 and osteoporosis by T-scores below -2.5. The diagnosis of severe osteoporosis entails a T-score below -2.5 and fragility fractures.

2.3.4 Primary osteoporosis

Rare bone disorders, that appear already in early childhood and have shared symptoms of osteopenia or osteoporosis and fragile bones that are subject to low-impact fractures, include osteogenesis imperfecta (OI), osteoporosis-pseudoglioma syndrome (OPPG) and non-OI primary osteoporosis. The three disorders can be distinguished from each other by their clinical symptoms, differing genetic causes or mode of inheritance.
**Osteogenesis imperfecta**

OI is a rare inherited disorder with a prevalence between 6 to 7 per 100,000 (Van Dijk *et al.* 2010). OI was classified in the late 1970s based on clinical and radiological findings and mode of inheritance (Sillence & Rimoin 1978, Sillence *et al.* 1979). Since the increase in molecular knowledge, the original classifications have been re-evaluated and modified (Van Dijk *et al.* 2010), and currently OI is divided into nine subtypes with varying clinical symptoms, genetic causes and/or inheritance patterns.

OI is a connective tissue disorder that is caused by defects in the structure of the collagen network (Basel & Steiner 2009). The common findings in OI types are osteoporosis and low-impact fractures. In addition, each subtype has a distinctive or slightly overlapping selection of other connective tissue related features such as bone deformities, short stature, blue sclera, dentinogenesis imperfecta, joint-laxity and hearing loss (Baujat *et al.* 2008). The severity and age of onset in OI ranges from perinatal lethal (type II) to mild osteoporosis observed only at adulthood (type I), though in most OI types the symptoms appear in early childhood (Basel & Steiner 2009, Michou & Brown 2011, Van Dijk *et al.* 2010). For a detailed review of OI types please see (Basel & Steiner 2009).

The severity of OI depends on the genetic cause and type of mutation. Over 90% of autosomal dominant OI cases are caused by mutations in genes encoding the pro-α1 or pro-α2 chains of collagen I (*COL1A1* or *COL1A2*), and these are usually found in single families (Michou & Brown 2011). Collagen I mutations may impair the triple helical structure through chain exclusion or chain non-exclusion. The autosomal dominant forms of OI that are caused by *COL1A1* and *COL1A2* mutations include types I-IV, which can be distinguished based on clinical findings (Basel & Steiner 2009, Michou & Brown 2011, Van Dijk *et al.* 2010). OI type V is also inherited in an autosomal dominant pattern, but its genetic cause is still unknown. Autosomal recessive forms of OI include types VI-IX. The genetic factors for VI remain unknown, but VII, VIII and IX result from mutations in cartilage-associated protein (*CRTAP*), leprecan (*LEPRE1*) and cyclophilin B (*PPIB*), respectively (Cabral *et al.* 2007, Morello *et al.* 2006, Van Dijk *et al.* 2009). These genes encode components of the enzyme complex that hydroxylates proline at the Y position (gly-X-Y) of *COL1A1* (Basel & Steiner 2009, Michou & Brown 2011).
**Osteoporosis-pseudoglioma syndrome**

OPPG is also a rare genetic disorder that manifests as early-onset osteoporosis, fractures and disrupted eye development resulting in congenital or infancy-onset visual loss (Gong et al. 1996, Gong et al. 2001). OPPG patients have a reduced volume of cancellous bone, but osteoblast and osteoclast numbers on bone surfaces are normal (Gong et al. 2001). Collagen synthesis, calcium homeostasis, the levels of catabolic and anabolic hormones, and bone turnover are all unaffected in OPPG patients (Gong et al. 1996). In the US, the population-incidence of OPPG is estimated at 1 per 2 000 000 (Ai et al. 2005a).

OPPG is an autosomal recessive disorder that is caused by mutations in the low-density lipoprotein receptor-related protein 5 (LRP5) gene. Heterozygous carriers of LRP5 mutations also present reduced BMD and osteoporotic fractures, but they do not have visual impairment (Ai et al. 2005a, Gong et al. 2001). The eye phenotype is suggested to ensue from failed involution of the vitreal vasculature during gestation (Gong et al. 2001), which leads to vitreous fibrosis and contracture and therefore loss of vision (Ko et al. 1985, Zhu et al. 2000). The effect results possibly from impaired Norrin signalling caused by LRP5 mutations (Ai et al. 2005a). By contrast, the LRP5 mutations affect the bone phenotype by decreasing the activity of the canonical Wnt signalling pathway and hence reducing bone formation (Ai et al. 2005a, Ai et al. 2005b, Saarinen et al. 2010, Urano et al. 2009).

**Non-OI primary osteoporosis**

Non-OI primary osteoporosis is a rare bone condition that emerges in early childhood before puberty. The first symptoms include dispersed pain in spine, hips and feet, which impair movement and lead to an atypical gait (Dent & Friedman 1965, Rauch et al. 2000). The disorder is characterised by reduced BMD, recurrent vertebral compression fractures and/or metaphyseal fractures in long bones after minimal trauma (Gordon et al. 2008, Ward LM 2003). The new bone formed during fracture healing in patients is often osteoporotic, and this state was named “neo-osseous porosis” (Dent & Friedman 1965, Dent 1977). Because of its poor quality, the neo-osseous porotic bone is prone to subsequent fractures caused by mechanical loading and physical exertion, but also by the strain caused by skeletal growth (Rauch et al. 2000).
The pathogenesis of non-OI primary osteoporosis is not well known. Rauch et al. (2000) described the histomorphology and suggested a pathogenic model for the disease development. Samples taken from patients showed reduced cancellous bone volume resulting from a diminished number of trabeculae and decreased trabecular thickness (Rauch et al. 2000). In addition, the BMUs responsible for bone remodelling were few and had impaired activity leading to a reduced bone formation rate. As a result, the formation of new cancellous bone was diminished and consequently the defective bone was not capable of responding and adapting to mechanical loading and strain during skeletal growth. In short, the disorder was suggested to develop as a consequence of impaired cancellous bone formation caused by the reduced rounds of remodelling cycles and decreased bone formation during each cycle (Rauch et al. 2000). In a subsequent study Rauch et al. (2002) specified that bone modelling of patients is mostly affected in the endocortical surface on the internal cortex i.e. cancellous surfaces that are in contact with the bone marrow environment (Rauch et al. 2000). The first studies on non-OI primary osteoporosis presumed it to be a non-hereditary disorder (Houang et al. 1978, Teotia et al. 1979), and Hartikka et al. (2005) were the first to detect a genetic component in the disease, namely the LRP5 gene.

2.3.5 Osteopetrotic conditions and HBM disorders

In addition to osteoporosis and osteopenia, disturbances in bone remodelling may lead to disorders with a completely opposite phenotype namely osteopetrosis and HBM disorders. These disorders result either from decreased osteoclastic function or increased osteoblastic activity.

Osteopetroses form a heterogenous family of bone disorders that present increased BMD caused by disrupted osteoclast function and defective bone resorption (Balemans et al. 2005). Osteopetrotic forms range from a mild form to a form that is fatal in infancy. They are classified into three subgroups based on their severity, inheritance pattern, age of onset and secondary features: autosomal recessive infantile malignant osteopetrosis (ARO), autosomal recessive intermediate mild osteopetrosis (IRO) and autosomal dominant adult onset benign osteopetrosis (ADO) (Stoker 2002). ARO is the most severe form of osteopetrosis and has an incidence of 1 per 250 000 (Del Fattore et al. 2008) The consequent symptoms are various, and encompass haematological deficiency, anaemia, cranial nerve compression with visual and hearing impairment, sclerotic and brittle bones and/or bone deformities (Del Fattore et al. 2008, Michou & Brown
In over 50% of cases loss-of-function mutations in the a3 subunit of the vacuolar H+ -ATPase of the ruffled border (TCIRG1) are observed, while mutations in Ruffled border Cl− conductance (CLCN7) and Ostm1 protein genes (OSTM1) account for 10% of cases (Michou & Brown 2011). The less severe forms of ARO are associated with mutations in genes such as RANKL, which induces osteoclast development (Sobacchi et al. 2007).

IRO is a rare intermediate form of osteopetrosis with a small number of cases caused by carbonic anhydrase type II (CAII) or Pleckstrin homology domain-containing protein family member M 1 (PLEKHMI) mutations (Michou & Brown 2011). Patients with CAII mutations also present renal tubular acidoses, cerebral calcifications and mental retardation (Kida et al. 2006). ADO is the most frequently met form of osteopetrosis with an incidence of 5:100 000 (Bollerslev & Andersen 1988). It is also the most heterogeneous class of osteopetrosis (Del Fattore et al. 2008), and is divided into three types, two of which (ADO II and III) are classified as osteopetroses and identified with dominant negative mutations on CLCN7 (Balemans et al. 2005). Interestingly ADO I associates with gain-of-function mutations in LRPS that, as opposed to the resorption deficiency in osteopetroses, result in increased bone formation by osteoblasts and is therefore categorised as a HBM disorder (Pangrazio et al. 2011, Wesenbeeck et al. 2003).

HBM disorders have phenotypic similarity to osteopetrosis, but the pathogenesis involves defective osteoblastic function. HBM patients present various clinical features such as increased BMD (Z-score > 4), high levels of alkaline phosphatase, entrapment neuropathies, torus palatinus and square jaw (Boyden et al. 2002, Little et al. 2002, Wesenbeeck et al. 2003). In addition to ADO I, HBM disorders include osteosclerosis (e.g. van Buchem disease) and hyperostosis (Wesenbeeck et al. 2003). The autosomal dominant form of HBM is caused by gain-of-function mutations in LRP5 (Boyden et al. 2002, Little et al. 2002, Wesenbeeck et al. 2003), which affect the activity of the canonical Wnt signalling pathway and consequently bone formation (Ai et al. 2005b, Balemans et al. 2007, Balemans et al. 2008, Boyden et al. 2002). Mutations in sclerostin (SOST), an inhibitor of the Wnt pathway, are also associated with sclerosteosis (Balemans et al. 2001, Brunkow et al. 2001, Pitters et al. 2010) and a deletion in the gene’s promoter causes van Buchem disease (Balemans et al. 2001, Loots et al. 2005, Staehling-Hampton et al. 2002).
2.4 Bone and canonical Wnt signalling pathway

Wingless-related murine mammary tumour virus (MMTV) integration site family members (Wnts) are secreted glycoproteins that are essential during embryogenesis (Wodarz & Nusse 1998), and 19 different Wnts have been identified in the human genome (He 2003) (http://www.stanford.edu/rnusse/wntwindow.html). They participate in a number of different processes such as embryonic induction, generation of cell polarity, specification of cell fate, and are also implicated in the genesis of cancer (for reviews see (Cadigan & Nusse 1997, Lustig & Behrens 2003)). Besides their expression during embryogenesis, Wnts are expressed in a variety of adult tissues including lymphoid tissues, colon, skin, hair follicles and bone (Alonso & Fuchs 2003, Bienz & Clevers 2000, Staal & Clevers 2003).

The Wnt signalling pathway promotes bone formation at several stages (Krishnan et al. 2006). Wnts are present at all stages of skeletal development (for a review see (Huelsken & Birchmeier 2001)) and they are expressed in osteoblasts (Glass DA,2nd & Karsenty 2006, Bodine & Komm 2006, Kalajzic et al. 2005). Canonical Wnt signalling directs the differentiation of mesenchymal stem cells into the osteoblastic lineage and also bone mineralisation (Day et al. 2005, Guo & Cooper 2007, Hill et al. 2005, Hu et al. 2005), but it is also necessary for osteoclast differentiation (Glass DA,2nd & Karsenty 2006). The canonical Wnt signalling pathway has been shown to affect postnatal bone formation in humans (for reviews see (Milat & Ng 2009, Westendorf et al. 2004)).

The Wnt signalling pathway is an intricate cascade activated by the presence and specific binding of Wnts on receptors located on the outer cell membrane. Previously Wnts were considered to bind only Frizzled (Fzd) receptors (Wodarz & Nusse 1998), but LRP5/6 co-receptors were found necessary for the signalling in the early 2000s (Pinson et al. 2000, Tamai et al. 2000, Wehrli et al. 2000). When Wnt is bound by the Fzd/LRP5 complex, the LRP5 protein’s cytoplasmic tail is phosphorylated (Zeng et al. 2005) and can bind yet another protein complex formed by the axis inhibitor (Axin), adenomatous polyposis coli (APC), Dishevelled (Dsh) and β-catenin, which is connected with glycogen synthase kinase 3-β (GSK3β) (Mao et al. 2001, Tamai et al. 2004). Further on in the process, the latter protein complex dissociates when the GSK3β-binding protein (GBP) removes GSK3β and β-catenin is released into the cytoplasm (Farr et al. 2000, Li et al. 1999). Consequently β-catenin accumulates in the cytoplasm and when the amount of unbound β-catenin exceeds the appropriate level, it enters the...
nucleus where it affects the transcription of Wnts target genes via transcription factors Lef1/Tcf (Billin et al. 2000, Shtutman et al. 1999). The pathway is not activated in the absence of Wnts, hence β-catenin is delivered to proteasomes for degradation and the level of β-catenin remains rather low (Aberle et al. 1997). For an illustration of the activated pathway see Figure 3A.

The pathway is repressed by inhibitors that prevent the interaction between Wnt ligands and their receptors (Figure 3B). Inhibitors like Dickkopfs (DKKs) and sclerostin (SOST) bind to LRP5/6 and remove it from the cell membrane making it unavailable for Wnts (Bafico et al. 2001, Li et al. 2005, Mao et al. 2002, Semenov et al. 2005), while secreted frizzled related proteins (sFRP) and the Wnt inhibitory factor (Wif-1) bind directly to Wnts and compete with receptor binding (Chung et al. 2004, Kawano & Kypta 2003).

Fig. 3. Illustration of the canonical Wnt signalling pathway A) the binding of Wnt proteins on LRP5 and Frizzled receptors activates the pathway. As a result, β-catenin escapes the protein complex, enters the nucleus and promotes the transcription of target genes. B) the activity of the pathway is disrupted by binding of inhibitors on LRP5 (SOST in I) and/or on Kremen (DKK1 in II). Upon inhibition, β-catenin is degraded and consequently transcription of target genes is prevented.
2.5 Bone and serotonin pathway

The groundbreaking discovery of Yadav et al. (2008) that Lrp5 regulates bone formation via control of serotonin (5-hydroxytryptamine i.e. 5-HT) production opened up new avenues of research into Lrp5 and bone biology. Since then the subject has been actively studied and debated, and knowledge has accumulated during the past few years.

The original observation was that Lrp5 expressed in the duodenal enterochromaffin cells could control the production of tryptophan hydroxylase-1 (Tph1), which is the rate-limiting enzyme for 5-HT production. By inhibiting Tph1 expression Lrp5 reduced the amount of circulating 5-HT and therefore promoted bone formation. This was supported by mouse models presenting gut-specific activation or inactivation of Lrp5 leading to increased or decreased bone formation, respectively. The effect of circulating 5-HT on osteoblasts was mediated through the 5-hydroxytryptamine receptor 1B (5-Htr1b) and 5-HT decreased osteoblast proliferation by reducing the production of the regulator of cell proliferation CyclinD1 via cAMP response element-binding protein (CREB) (Figure 4). (Yadav et al. 2008)

**Fig. 4.** Schematic presentation of duodenal Lrp5’s effect on Tph1 expression according to Yadav et al. (2008). Reduced expression of Lrp5 in the intestine increases Tph1 expression and consequently 5-HT production. 5-HT enters the circulatory system and binds to 5-Htr1b receptors on osteoblasts, hence reducing the amount of Creb and therefore leading to curtailed cell proliferation and bone formation. The schematic is modified from Warden et al. (2010).
Ninety-five per cent of total 5-HT is produced in the intestine (Gershon & Tack 2007), but 5-HT is also expressed in the brain, controlled by Tph2 (Walther et al. 2003). As opposed to gut-derived 5-HT, the brain-derived isoform has a dominant role and it promotes bone accrual (Yadav & Karsenty 2009, Yadav et al. 2009). The expression of brain-derived 5-HT, and consequently bone formation, is inhibited by leptin, the hormone commonly associated with weight and energy expenditure control (Ducy et al. 2000, Oury et al. 2010, Shi et al. 2008, Yadav & Karsenty 2009, Yadav et al. 2009). Indeed, knockout mice of leptin (ob/ob) or its receptor (dp/dp) are overweight and develop an HBM phenotype (Ducy & Karsenty 2000). In contrast, mice lacking Tph2 (Tph2-/-), and hence deficient in 5-HT, present with osteopenia and anorexia although they have normal levels of leptin, insulin and corticosteroids (Yadav et al. 2009).

In clinical studies increased serum 5-HT levels have been observed in OPPG patients with LRP5 mutations (Saarinen et al. 2010, Yadav et al. 2010a), while patients with HBM phenotype causing LRP5 mutations have had decreased 5-HT levels (Frost et al. 2010, Yadav et al. 2008). From a medicinal aspect, treatment of ovariectomised or Lrp5 deficient mice that have age-related bone loss have shown promising results. Reduced bone mass in these mice has been improved after medicating with Tph1 (Inose et al. 2011) or 5-HT (Yadav et al. 2010b) inhibitors. Meanwhile, the results on selective serotonin-reuptake inhibitors (SSRIs) that inhibit the 5-HT transporter (5-HTT) have been contradictory as some studies have shown deleterious effects on bone both in clinical (Haney et al. 2007, Liu et al. 1998, Richards et al. 2007, Weintrob et al. 2002) and mice studies (Bonnet et al. 2007, Warden et al. 2005, Warden et al. 2008) while some have shown the opposite effects (Battaglino et al. 2007, Gustafsson et al. 2006). For a review on SSRIs and their clinical outcome in bone please see (Haney & Warden 2008).

It is still possible that Lrp5 affects bone formation by osteoblasts and osteocytes in response to mechanical loading (Robinson et al. 2006, Sawakami et al. 2006). In fact, results inconsistent with those of Yadav et al. (2010a) were obtained in a recent study proving altered bone mass in mice with osteocyte specific activation or inactivation of Lrp5, that was not observed in gut specific transgenic mice (Cui et al. 2011). Furthermore, the bone specific Lrp5 genotype did not affect 5-HT synthesis, nor did the serum 5-HT levels correlate with the Lrp5 genotype or bone mass (Cui et al. 2011). Furthermore, treating ovariectomised mice with a Tph1 inhibitor decreased the circulating 5-HT levels, but no effect on bone formation markers or bone mass in femur or vertebrae was
observed (Cui et al. 2011). These results were supported by a subsequent study in which mice with HBM caused by Lrp5 or Sost mutations showed no difference in the serum serotonin levels as compared to WT mice (Niziolek et al. 2011).

2.6 Inheritance of bone characters/quality

Skeletal development and health entails complex processes and strict control that rely on both inherited and environmental factors. BMD is under strong genetic control and is often used as a hallmark in studying the genetics of bone (Giroux et al. 2008). Twin and family studies have proved a significant genetic effect in determining bone parameters and estimates of the genetic component of BMD range between 50–80% (Eisman 1999, Stewart & Ralston 2000). Genetic effects also partially account for changes in BMD (Kelly et al. 1993). Furthermore a family-history of low BMD, fractures or osteoporosis are known risk factors for reduced bone parameters and disorders (Danielson et al. 1999, Grainge et al. 1999, Kanis et al. 2004, Seeman et al. 1994).

2.6.1 Genetics of osteoporosis

Osteoporosis is a multifactorial disease with both genetic and environmental components. Individual genes may contribute to the risk of osteoporosis, but also gene-gene interactions and/or gene-environment interactions play a role. This makes the study of osteoporosis a challenging task. Candidate gene association studies of rare monogenetic bone disorders (such as those described in sections 2.3.4. and 2.3.5.) have been instrumental in resolving some of the genetic components in osteoporotic conditions. Among the most studied candidate genes for fractures and osteoporosis are those that encode proteins involved in maintaining calcium homeostasis (Vitamin D receptor (VDR), Parathyroid hormone (PTH), PTH receptor, Calcitonin (CT), CT receptor (CTR), Calcium-sensing receptor, Glucocorticoid receptor) and bone composition (COL1A1, COL1A2, Osteopontin, Osteocalcin, Osteonectin), regulate bone growth (Aromatase (CYP19A1), Estrogen receptors α and β (ER or ESRα and -β), Androgen receptor (AR), Interleukin-6 (IL-6), Insulin-like growth factor 1, Transforming growth factor β1, Bone morphogenetic proteins 2, 4 and 7 (BMP-2, -4 and -7) and/or function in signalling pathways (LRP5, LRP6, Receptor activator of nuclear factor κB (RANK), RANKL, SOST) (for reviews please see (Lei et al. 2007, Marini & Brandi 2010)).
More than 60 quantitative trait loci (QTL), spread over all chromosomes apart from Y, have been associated with bone metabolism in genome-wide association analyses (GWAS) (Liu et al. 2006). The first GWAS study performed showed weak associations for BMD with loci close to e.g. ER$\alpha$, CYP19, COL1A1, VDR and LRP5, while the majority of the top 40 loci were located near new candidate genes for osteoporosis, however the findings were not significant genome-wide (Kiel et al. 2007). Since then the list has been extended with loci near to or within RANKL, OPG and ESR1 (Styrkarsdottir et al. 2008), and with LRP5 and OPG which showed a significant association with BMD and marginally with fractures in another study (Richards et al. 2008). During the last few years meta-analyses have been performed to improve the power of GWAS. One of these studies led to the detection of Microtubule affinity-regulating kinase 3 (MARK3), SOST and Major-histocompatibility-complex with strong associations with BMD (however not at genome-wide levels) (Rivadeneira et al. 2009), while another large meta-analysis revealed new genome-wide associations in SOST, Specificity protein 7, RANK and MARK3 with suggestive results at LRP4, Disintegrin and metalloproteinase domain 19, Integrin-binding sialoprotein and C17orf53 (Styrkarsdottir et al. 2009).

2.6.2 Candidate genes in stress fractures

The role of genetic factors in predisposing to stress fractures is supported by twin and family studies. Monozygotic twins have been reported with multiple stress fractures at identical anatomical sites during military service (Singer et al. 1990), and other studies have presented identical or highly similar sport-related fractures in monozygotic twin pairs (Murray et al. 2005, Reina et al. 2010, Van Meensel & Peers 2010). Furthermore, multiple lower limb stress fractures have been observed in the same individual indicating a high susceptibility for stress fractures (Lambros & Alder 1997).

The search for the genetic factors of stress fractures is just beginning since so far only three studies have been reported (Chatzipapas et al. 2009, Välimäki et al. 2005, Yanovich et al. 2011). In the first article two polymorphic sites (XbaI, PvuII) on the ER (or ESR1) and the CAG repeat from the AR were studied from 15 Finnish military conscripts with stress fractures, but no associations were observed (Välimäki et al. 2005). A pilot study by Yanovich et al. (2011) found an association between AR CAG repeat length and stress fractures, the risk being halved in military conscripts who had less than 16 repeats. Chatzipapas et al.
(2009) assessed four polymorphic sites from the VDR gene (FokI, BsmI, ApaI and TaqI) in male military personnel and found increased risk for stress fractures and association with reduced T-scores in F (presence of FokI restriction site) and B alleles (absence of BsmI restriction site). As the knowledge on genetic factors in stress fractures is still sparse, the candidate genes have been selected based on the information available from studies on the genetics of bone parameters such as BMD.

Collagen I forms a major part of the organic component in bone (Robey & Boskey 1996), hence the encoding gene has been an evident choice for candidate gene analysis. It is known that mutations in COL1A1 or COL1A2 cause OI (Körkkö et al. 1998), and polymorphisms in COL1A1 associate with BMD, low bone density, osteoporosis and increased risk for vertebral fractures (Garnero et al. 1998, Grant et al. 1996, Uitterlinden et al. 1998).

Polymorphisms in the genes encoding three receptors, VDR, the CTR and ESR1, have been implicated in BMD and/or bone pathologies. VDR is a crucial receptor for 1,25(OH)2D3 to mediate its calcemic and phosphatemic effects on bone remodelling and mineralisation (Haussler et al. 1998). Morrison et al. (1994) were the first to identify a correlation between the VDR genotype and bone mass, which led to a search for genetic factors predisposing to osteoporosis. The studies that ensued have shown associations between VDR polymorphisms and low BMD and osteoporotic fractures (Horst-Sikorska et al. 2007, Uitterlinden et al. 2001), but the results have not been strictly consistent (Eisman 1999, Li et al. 2010).

CTR also has a central role in sustaining bone homeostasis, and several studies have shown CTR genotypes to associate with BMD values (Braga et al. 2002, Masi et al. 1998a, Masi et al. 1998b, Masi et al. 2002, Nakamura et al. 2001, Tsai et al. 2003). Furthermore estrogens are important regulators of bone metabolism and therefore their receptors play a central role. ESR1 became a promising candidate gene for osteoporosis after its inactivation was observed to associate with low BMD (Korach 1994, Smith et al. 1994), and since then several studies have shown ESR1 polymorphisms to correlate with either low BMD and/or osteoporotic fractures (Becherini et al. 2000, Greendale et al. 2006, Kobayashi et al. 1996, Langdahl et al. 2000, Moron et al. 2006).

Finally, IL-6 participates in bone metabolism and is suggested to increase osteoclast activity in bone diseases (Manolagas 1998, Roodman et al. 1992, Roodman 1995), while OPG regulates bone resorption by reducing osteoclastogenesis and osteoclast differentiation (Simonet et al. 1997). Polymorphisms in IL-6 have been associated with BMD (Lorentzon et al. 2000,
Murray et al. 1997), and variations in OPG associate with osteoporosis (Arko et al. 2002, Ohmori et al. 2002) and mutations with e.g. Paget’s disease (Whyte et al. 2002).

2.6.3 The LRP5 gene mutations

LRP5 is a transmembrane protein of 1615 amino acids (Dong et al. 1998, Hey et al. 1998) that belongs to the superfamily of low-density lipoprotein receptor cell-surface receptors (Brown et al. 1997, Krieger & Herz 1994). The human chromosomal region of 11q12-13 was first assigned with bone disorders OPPG, HBM and autosomal recessive osteopetrosis in the late 1990s (Gong et al. 1996, Heaney et al. 1998, Johnson et al. 1997, Koller et al. 1998). Cloning and characterisation of LRP5 was performed a few years later (Dong et al. 1998, Hey et al. 1998), and the first mutations were identified in patients with OPPG (Gong et al. 2001). A schematic picture of the LRP5 structure is presented in Figure 5.

Fig. 5. Schematic presentation of LRP5 protein. LRP5 contains four propeller domains that each encompass five YWTD motifs and an epidermal growth factor (EGF) repeat. Furthermore, LRP5 has three low density lipoprotein (LDL) repeats, a transmembrane domain and a cytoplasmic domain.

Mutations in LRP5 have been associated with varying bone disorders for about a decade. Homozygous and/or compound heterozygous loss-of-function mutations were first associated with OPPG (Ai et al. 2005a, Gong et al. 2001, Kato et al. 2002) and soon thereafter dominant heterozygous gain-of function mutations on LRP5 were identified in patients with HBM conditions (Balemans et al. 2007, Balemans et al. 2008, Boyden et al. 2002, Little et al. 2002, Wesenbeeck et al. 2003). The majority of the disease-associated mutations are single nucleotide changes in coding regions of LRP5 leading to missense or nonsense mutations, but lately trinucleotide deletions in the signal sequence (Chung et al. 2009) and mutations affecting splicing sites (Laine et al. 2011) have been recognised in OPPG patients. The mutations in LRP5 are presented in Supplemental table 1. In addition to mutations, LRP5 polymorphisms have been associated with bone parameters, BMD and quality (Urano et al. 2009) both in males (Ferrari et al.
2004) and females (Giroux et al. 2007, Giroux et al. 2008). Furthermore, LRP5 variants have been shown to contribute to the risk of osteoporosis (Mizuguchi et al. 2004), fractures (Bollerslev et al. 2005) and idiopathic osteoporosis in males (Ferrari et al. 2005).

The significance of Lrp5 in bone has been supported by mouse studies, which show a high resemblance between the corresponding mouse and human bone phenotypes. Lrp5 deficient mice (Lrp5−/−) develop osteopenia resulting from defective osteoblast proliferation and function, but they also present impaired eye vascularisation similar to OPPG patients (Kato et al. 2002). Mice with compound mutants of Lrp6 and Lrp5 (Lrp6−/−; Lrp5−/− and Lrp6+/−; Lrp5+/−) suffer from limb deformities and reduced BMD (Holmen et al. 2004), while mice with HBM mutations have increased BMD and bone parameters (Babij et al. 2003, Cui et al. 2011) resulting from increased osteoblast number and their increased lifespan and function (Babij et al. 2003, Niziolek et al. 2011). Furthermore, HBM mutations and Wnt signalling were shown to have a local effect on bone in Lrp5 knock-in mice with HBM mutations (Cui et al. 2011). Interestingly, a recent mouse study showed variation between bone phenotypes caused by different Lrp5 HBM mutations (Niziolek et al. 2011). The Lrp5 mutant A214V (resembling the phenotype of the Sost knock-out) led to increased periosteal bone formation by presumably affecting Sost binding while the G171V mice increased endocortical bone formation via an unknown mechanism. The possible reasons for this effect of the G171V mutation include variable affinity for different Wnt ligands or altered trafficking to the cell membrane. The finding is remarkable when one considers that these HBM mutations are located in the same exon and in the same binding pocket (Niziolek et al. 2011). The result suggests separate mechanisms of action for individual mutations causing similar phenotypes (Niziolek et al. 2011).

Along with mouse models, in vitro studies have been carried out in the search for the underlying mechanisms of LRP5 mutations. Commonly HBM mutants are able to reach the cell membrane and mediate Wnt signalling (Ai et al. 2005b, Balemans et al. 2007), and only one HBM (M282V) mutation has been reported for decreased protein production (Balemans et al. 2007). Instead, HBM mutations have been reported with reduced interaction and inhibition by DKK1 (Ai et al. 2005b, Balemans et al. 2007, Balemans et al. 2008, Boyden et al. 2002), but also by SOST (Balemans et al. 2008, Semënov & He 2006). In addition, one publication has suggested an impaired binding of Mesd to HBM- G171V, the signalling activity being retained through autocrine Wnt signalling (Zhang et al. 2004). In vitro studies on OPPG-associated mutations have shown that some of
these mutations affect protein trafficking to the cell membrane while others are incapable of mediating Wnt1 or Wnt10b signalling or have reduced signalling capacity (Ai et al. 2005a, Chung et al. 2009). Deletions or splicing mutations in LRP5 may lead to defects in protein function, truncation of protein product or disruption of intracellular protein trafficking resulting in impaired signalling (Chung et al. 2009, Laine et al. 2011).

### 2.6.4 Components of the canonical Wnt signalling pathway in bone

The role of the canonical Wnt signalling pathway in bone biology has been acknowledged, and polymorphisms in genes encoding several components of the pathway have been associated with BMD in subjects with low or high bone mass (Sims et al. 2008). Also mutations in these genes have been reported to be related to bone disorders. The components of the Wnt pathway selected for the candidate gene analyses of non-OI primary osteoporosis in this thesis are presented in more detail in the current chapters.

**Inhibitors**

The family of Dickkopf proteins (Dkks) contains four members, of which DKK1 is the best studied and influential in bone biology along with DKK2. These two Dkks have been shown to control the mineralisation process of osteoblasts: Dkk1 is involved in the early phases of osteoblastic development and Dkk2 participates in orchestrating osteoblast proliferation and maturation (van der Horst et al. 2005). Multiple myeloma (MM) is an osteolytic bone disorder that manifests in bone pain, fractures and hypercalcemia (Heath et al. 2009). Osteolytic bone lesions ensue from interactions between myeloma cells and the bone-marrow microenvironment (Haaber et al. 2008), and are caused by increased osteoclast activity and decreased osteoblast activity (Bataille et al. 1989). Wnt signalling participates in this by inhibiting osteoclastogenesis in bone marrow (Qiang et al. 2008a, Qiang et al. 2010) while DKK1 represses osteoblastogenesis (Qiang et al. 2008a, Tian et al. 2003). DKK1 seems to be one of the key players in MM for it is overexpressed in MM patients (Tian et al. 2003) and the severity of osteolytic bone lesions correlates with DKK1 levels (Haaber et al. 2008). Promising results have been obtained using DKK1 antibodies suggesting therapeutic applications for treating or preventing bone osteolytic lesions and MM (Glantschnig et al. 2010, Heath et al. 2009, Heiland et al. 2010, Yaccoby et al. 2007).
Mouse studies also support the role of Dkk1 and -2 in bone. Loss of one Dkk1 allele (Dkk1\textsuperscript{+/-}) leads to increased bone mass (Morvan \textit{et al.} 2006) and Dkk1 overexpression produces osteopenia (Li \textit{et al.} 2006). A direct effect of Dkk1 on bone was suggested in a study showing that widespread overexpression of Dkk1 results in osteopenia along with alopecia and forelimb defects while Dkk1 overexpression targeted specifically to bone caused severe osteopenia with normal limb and hair growth (Li \textit{et al.} 2006). Mice with differing Dkk1 genotypes (Dkk1\textsuperscript{+/-}, +/+ or d/-) and consequently Dkk1 expression levels showed bone mass to be inversely proportional to the prevailing amount of Dkk1 (MacDonald \textit{et al.} 2007). In addition, the reduced bone mass of ovariectomised rats was restored partly by knocking down Dkk1 (Wang \textit{et al.} 2007). Curiously, Dkk2\textsuperscript{-/-} mice are osteopenic as a consequence of poor bone mineralisation resulting from defective osteoblast activity, but they also have an increased number of osteoclasts suggesting a role for Dkk2 both in osteoblast and osteoclast differentiation (Li \textit{et al.} 2005).

Loss-of-function mutations in \textit{SOST} are associated with sclerosteosis, (Balemans \textit{et al.} 2001, Brunkow \textit{et al.} 2001, Pitors \textit{et al.} 2010) and a 52-kb deletion of an important regulatory element downstream of \textit{SOST} causes van Buchem disease (Balemans \textit{et al.} 2001, Loots \textit{et al.} 2005, Staehling-Hampton \textit{et al.} 2002). Also, polymorphisms on \textit{SOST} have been associated with BMD (Uitterlinden \textit{et al.} 2004). SOST, an inhibitor of the Wnt signalling pathway, is expressed solely in osteocytes in adult bone (van Bezooijen \textit{et al.} 2005) and mutations in \textit{SOST} or \textit{LRP5} impair their interactions and hence affect the activity of the pathway and bone formation (Balemans \textit{et al.} 2008, Ellies \textit{et al.} 2006, Semenov & He 2006) Accordingly, SOST\textsuperscript{-/-} and SOST-knockout (KO) mice have HBM (Li \textit{et al.} 2008, Loots \textit{et al.} 2005), mice over-expressing SOST present reduced bone mass and strength (Winkler \textit{et al.} 2003), and fracture healing is enhanced in SOST-KO mice due to increased bone formation (Li \textit{et al.} 2011, Li \textit{et al.} 2010). As with DKK1, sclerostin antibodies have also been tested for therapeutic implementation: sclerostin antibody increased bone formation and reduced resorption in male (Li \textit{et al.} 2010) and female rats with induced osteopenia (Tian \textit{et al.} 2010, Tian \textit{et al.} 2011), and accelerated fracture healing in aged male rats and cynomolgus monkeys (Li \textit{et al.} 2010).

Axin1 and -2 are highly similar in function, but they differ in expression as Axin1 is expressed in nearly all tissues while Axin2 expression is more restricted and varies between tissues and developmental stages (Chia & Costantini 2005). Mutations in both genes have been implicated in colon cancer (Jin \textit{et al.} 2003,
Axin1 is crucial for normal axis development: deletion of Axin1 in mouse results in early embryonic mortality caused by neural defects and axis duplication (Zeng et al. 1997), and in humans AXIN1 mutations cause caudal duplication (Kroes et al. 2002). AXIN2 mutations are associated with impaired tooth development (Lammi et al. 2004) and loss of Axin2 in mouse causes skull defects during development (Yu et al. 2005). Bone formation was highly increased in Axin2-KO mice leading to an HBM phenotype because of increased osteoblast and reduced osteoclast activity resulting from enhanced Wnt signalling (Yan et al. 2009). Axin2LacZ/LacZ mice, that have increased sensitivity to Wnt signalling, present accelerated recovery from bone injury because of induced osteoblast differentiation (Minear et al. 2010), the effect being further enhanced by targeting sites of injury with Wnt3a packaged liposomes (Minear et al. 2010).

Activators

Wnt3a participates in controlling the development of the apical ectodermal ridge during chick limb patterning (Kengaku et al. 1998). It induces osteoblastogenesis and inhibits adipogenesis via controlling the expression of genes that affect these events (Jackson et al. 2005, Rawadi et al. 2003). Furthermore, Wnt3a activates the expression of genes encoding components of the non-canonical Wnt signalling pathway (Qiu et al. 2011), and indirectly stimulates the production of phosphatidylinositol 4,5-bisphosphate, which participates in the regulation of LRP6 phosphorylation (Pan et al. 2008, Qin et al. 2009). Finally, an expression array performed at certain differentiation stages of osteoblasts revealed that Wnt3a expression increased in mature osteoblasts (Kalajzic et al. 2005).

Wnt10b regulates bone growth by promoting osteoblastogenesis and inhibiting adipogenesis (Bennett et al. 2005, Ross et al. 2000). Mice expressing Wnt10b in marrow (FABP4-Wnt10b) develop increased bone mass and strength, and are more resistant to age-related bone loss than control mice (Bennett et al. 2005, Bennett et al. 2007) as a consequence of increased osteoblast activity (Bennett et al. 2007). In contrast, Wnt10b deficient (Wnt10b−/−) mice present reduced trabecular bone and serum osteocalcin levels (Bennett et al. 2005, Bennett et al. 2007). A modest association between two SNPs in WNT10B and lowered hip BMD and bone structure was shown in a population- and family-based study of African origin (Zmuda et al. 2009).
3 Outlines of the present study

Osteoporosis is a bone disorder that leads to a reduction in bone volume, deterioration of bone microarchitecture and therefore increased fracture risk. Bone disorders like osteoporosis are affected by both environmental and genetic factors. Family and twin studies have shown the importance of genetics in bone formation and health, but most of the genetic factors contributing to bone formation are still largely unknown. The aim of this thesis was to search for and identify genetic factors that predispose to disorders of reduced bone mass and to elucidate their importance and mechanism of action in in vitro studies. The study was carried out by approaching two bone disorders that manifest in bone fragility and fractures: femoral neck stress fractures and non-OI primary osteoporosis. Because the canonical Wnt signalling pathway is crucial for normal bone formation, and mutations in the genes encoding for components of the pathway (e.g. LRP5, DKK1 and SOST) result in several different bone disorders, the main focus on the research was to study the role of these components at the genetic and cellular levels. Identifying mutations in the genes affecting skeletal development and studying the effects of these mutations is important because the aetiology of bone disorders is not well understood. Our genetic studies of bone disorders add valuable information on the factors predisposing to bone fragility and fractures, which enhance our knowledge on diagnostics of bone disorders and promote the development of effective treatment.

The specific aims were:

1. to search for candidate genes affecting the development of femoral neck stress fractures in young Finnish men
2. to study the role of the LRP5 gene mutations and their functional importance in non-OI primary osteoporosis
3. to search for putative disease causing mutations from eight candidate genes selected from components of the canonical Wnt - and serotonin signalling pathways in non-OI primary osteoporosis
4 Material and methods

4.1 Patient and control sets (I-III)

A set of Finnish male conscripts with femoral neck stress fractures and two sets of non-OI primary osteoporosis patients (patient set I and II), along with their respective control groups, were used to study the genetic factors of these bone disorders that manifest in severe fractures.

4.1.1 Military conscripts with femoral neck stress fractures (I)

All military conscripts who had suffered from femoral neck stress fractures while performing their military service during 1970 to 1995 were invited to attend a follow-up examination. The follow-up was carried out in 2002 and 2003, and 72 conscripts were available and employed in the study. Accepted radiographic, scintigraphic or MRI criteria (Anderson & Greenspan 1996, Kiuru et al. 2004) were originally applied to diagnose the stress fractures. All subjects were male and their age of onset varied from 18 to 27 years. The control set was collected during the early 2000s and consisted of 120 conscripts with no history of femoral neck stress fractures before or during their military service according to military medical records and a personal questionnaire. The age of controls ranged from 18 to 22 years. In addition, a control set of clinical contemporaries of patients (127 healthy military conscripts) was applied in the study.

EDTA blood samples were taken from all participants and genomic DNA was extracted from peripheral blood lymphocytes using a standard procedure. Clinical information available from the military medical records included age, sex, height, weight, and smoking habit. Body mass index (BMI) was calculated based on the clinical information (weight divided by square root of height in metres i.e. kg/m²). The study was approved by the local ethics committee (Finnish Defence Forces, Helsinki, Finland), and signed informed consent was received from all participants.

4.1.2 Non-OI primary osteoporosis (II, III)

Two paediatric patient sets (patient set I and II) were used to search for putative disease causing mutations in patients with non-OI primary osteoporosis. Patient
set I, collected from Italy and Germany, included 18 patients analysed for the
**LRP5** gene (article II). Patient set II consisted of 15 Canadian patients, who were
screened for 8 candidate genes (**DKK1**, **DKK2**, **WNT3A**, **WNT10B**, **AXIN1**, **SOST**, **TPH1** and **5-HTR1B**) in the third article of the thesis. Patient set II had been
previously analysed for the **LRP5** gene in Hartikka et al. (2005), and patients
identified with **LRP5** mutations (OP5, OP8, OP16 (Hartikka et al. 2005)) were
excluded from further candidate gene analyses.

The patients had been referred to Paediatric bone health clinics and were
diagnosed by expert clinicians. All patients had a history of recurrent fractures of
long bones, bone pain, findings of osteopenia on imaging and/or low BMD. The
diagnostic criteria of non-OI primary osteoporosis included: I) clinical and
genetic exclusion of OI, II) exclusion of secondary causes of osteoporosis, and III)
low BMD, defined as Z-score below -2.0, history of recurrent peripheral fractures
(≥3 fractures) caused by low impact trauma, and/or findings of vertebral
affected and unaffected family members of the patients with mutations were
collected and genetic analyses were performed.

Controls were collected from the same geographical regions as the patients
who were found to have mutations or variants. These were identified from
German and Canadian patients hence control sets included 51 healthy German
and 80 Canadian controls with diagnoses of skeletal dysplasia (Schmid type of
metaphyseal dysplasia, multiple epiphyseal dysplasia or Ehlers-Danlos syndrome)
with no signs of osteoporosis. The study was approved by the local ethics
committee, and signed informed consent was obtained from all participants.

### 4.2 Genetic analyses (I-III)

#### 4.2.1 Polymerase chain reaction (PCR) (I-III)

Exons and their flanking regions were amplified using the polymerase chain
reaction (PCR) method. PCR primers were designed for **COL1A1** (51), **COL1A2**
(52), **OPG** (8), **ESR1** (5), **LRP5** (promoter and 23 exons), **DKK1** (promoter and 4
exons), **DKK2** (4), **WNT3A** (4), **WNT10B** (4), **AXIN1** (10), **SOST** (2), **TPH1** (10)
and **5-HTR1B** (1), with the number of exons indicated in parentheses. The size of
the PCR products ranged from 236 to 655 bp.
PCR reactions were performed in a total volume of 23 μl comprising 60 ng of genomic DNA, 1.5 mM MgCl₂, 0.23 mM of dNTPs, 5 pmol of each PCR primer and 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). For some exons the addition of dimethyl sulphoxide (DMSO) into the PCR reaction was necessary to optimise the quantity of the PCR product. These included exons 1 and 4 of WNT3A, exon 2 of WNT10B, and exons 1 and 2 of DKK2. For LRP5 exons 5 and 21 AmpliTaq Gold360 (Applied Biosystems) was used and exon 1 was amplified using the GC Rich PCR kit (Roche Applied Sciences). The PCR program used was the following: one cycle of 94.5 °C for 12 min, 35 cycles of 94.5 °C for 30 s, 53–65 °C for 30 s (depending on the PCR primers used) and 72°C for 30 s, and one cycle of 72 °C for 5 min. For CSGE analysis the program was extended by denaturation at 98.0 °C for 5 min and annealing at 68 °C for 30 min to produce heteroduplexes. PCR products were checked for quality and quantity by agarose gel electrophoresis (AGE).

4.2.2 Conformation sensitive gel electrophoresis (CSGE) (I, III)

Genes and the intronic boundaries were screened for genetic variation using conformation sensitive gel electrophoresis (CSGE) (Körkkö et al. 1998). Samples were amplified using PCR as described in Methods 4.2.1. For CSGE a mixture of different sized PCR products was prepared and 3 μl of 10× loading buffer (30% glycerol / 0.25% bromphenol blue / 0.25% xylene cyanol FF) was added to the samples before loading them into lanes. Glass plates of 37.5 × 45 cm were used to prepare a 1 mm thick CSGE gel. The gel consisted of 15% polyacrylamide (GE Healthcare), a 99:1 ratio of 1,4-bis(acryloyl) piperazine (BAP) (Fluka), 15% formamide (AMRESCO), 10% ethylene glycol (MERCK), 0.1% ammoniumpersulphate (SIGMA) and N,N,N’,N’-tetramethylethylenediamine (SIGMA) in 0.5×TTE buffer (containing 22% Tris (MP Biomedicals) / 7.2% taurine (USB) / 0.4% EDTA (VWR)). The gel was pre-electrophoresed for 20 min at 40 W, samples were loaded in the gel and electrophoresed first at 40 W for 30 min and then at 20 W for 10 hours. 0.5% TTE buffer was used in the electrophoresis chambers. The gel was removed from between the glass plates and stained with SYBR Gold (Invitrogen) and DNA bands were detected under a hand-held UV-light (UVP). The gel was cut, transferred for observation on a white / ultraviolet light transilluminator (UVP) and photographed with a charge-coupled device (CCD) camera (UVP).
**4.2.3 Multiplex ligation-dependent probe amplification (MLPA) (II)**

Multiplex ligation-dependent probe amplification (MLPA) (Schouten et al. 2002) was performed on non-OI primary osteoporosis samples to screen the \( LRP5 \) gene for deletions and/or insertions. The \( LRP5 \) and control (acetylcholinesterase i.e. \( ACHE \), exon 2) probes were synthesised by Integrated DNA Technologies (Coralville, IA). B probes were 5’-phosphorylated and contained non-specific stuffer sequences of varying lengths creating uniquely-sized amplicons in the amplification of each exon’s ligated probe pair.

Two MLPA reactions were performed for each genomic DNA sample: one using probes that targeted the even-numbered \( LRP5 \) exons (i.e. “Evens”) as well as the \( ACHE \) exon 2, and the other targeting the odd-numbered \( LRP5 \) exons (i.e. “Odds”). Dilutions of 100 ng of DNA in 5 μl TE buffer (pH 8) were heated at 98 °C for 5 min to denature each sample, then cooled to 25 °C. Probe annealing reactions were completed by adding 1.5 μl of the “Evens” or “Odds” probe mixture (probe mix containing 2 fmole of each probe) and 1.5 μl of salt solution (1.5 M KCl, 300 mM Tris-HCl pH 8.5, 1 mM EDTA) in each DNA tube (Schouten et al. 2002). For probe hybridisation, the reactions were incubated at 95 °C for 1 min 10 s and at 62 °C (“Evens”) or 61 °C (“Odds”) for 18 h. The reaction temperature was reduced to 54 °C, and 32 μl of Taq ligase buffer/enzyme mixture was added to the reactions to ligate the annealed probes. After 15 minutes of further incubation at 54 °C, the reactions were heated to 98 °C for 5 min, then cooled to room temperature and stored at -20 °C.

Amplification reactions were performed using PCR comprising 10 μl of ligation reaction, 0.75 mM MgCl\(_2\), 2.5 mM dNTPs, 0.1 μM MLPA-F (FAM-labelled), 0.1 μM MLPA-R and 2.5 U Taq (Invitrogen) in a total volume of 50 μl. The PCR program consisted of 34 cycles of 95 °C for 40 s, 60 °C for 30 s, and 72 °C 1 min, followed by 72 °C for 20 min. One PCR primer pair was used to amplify all probe ligations, as all probes contained the same primer target sequences. Amplicons were examined on 4% NuSieve 3:1 Agarose (Cambrex) to confirm amplification. Single amplicons could not be distinguished within the mix; each set of 12 amplicons showed a smear within the amplicon size range. Samples were prepared for analysis by adding 9.5 μl formamide and 0.5 μl of standard (ABI Liz500) to 1 μl of amplicon. Samples were injected on the ABI3100 (Applied Biosystems), on a 16-capillary-set, 36” long, with an injection time of 20 s, at the Case Western Reserve Genomics Core Facility (Case Western Reserve University School of Medicine, Cleveland, Ohio). The results were
analysed with GeneScan Analysis 3.7 (Applied Biosystems) and peak heights of amplicons were normalised against the ACHE peak (“Evens”) or against the LRP5 exon 15 (“Odds”). Deletions under the probe targets were identified based on reduced peak heights as compared with controls.

4.2.4 Sequencing (I-III)

Samples containing heteroduplexes on CSGE were further analysed by direct sequencing. Samples were first purified using Shrimp Alkaline Phosphatase (SAP) and Exonuclease I to remove nucleotide and primer residues. Sequencing was performed using an ABI PRISM 377 (Applied Biosystems) or 3100 Sequencer (Applied Biosystems) and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Kit (Applied Biosystems). GenBank accession numbers for candidate genes sequences in articles II and III were NG_015835.1 (LRP5), NM_012242.2 (DKK1), NM_014421.2 (DKK2), NM_033131.3 (WNT3A), NM_003394.3 (WNT10B), NM_181050.2 (AXIN1), NM_025237.2 (SOST), NM_004179.2 (TPH1) and NM_000863.1 (5-HTR1B).

4.2.5 Genotyping of 15 SNPs in the candidate genes (I)

Three SNPs from COL1A1 and COL1A2 and four SNPs from LRP5 were studied as described in Methods 4.2.1. Detailed information on the genotyped SNPs is presented in article I. Also three SNPs were genotyped from VDR (FokI, BsmI, TaqI) (Gross et al. 1996, Morrison et al. 1994, Riggs et al. 1995), and one each from IL6 (NlaIII) (Lorentzon et al. 2000) and CTR (AluI) (Nakamura et al. 2001). PCR products were digested with the respective restriction enzymes and the products were examined on 2–12% AGEs stained with ethidium bromide (Gross et al. 1996, Lorentzon et al. 2000, Morrison et al. 1994, Nakamura et al. 2001, Riggs et al. 1995). The genes that had shown biological gene interactions (Fretz et al. 2007, Gong et al. 2001, Peleg et al. 1993) and had large enough samples size were selected as candidates for gene-gene interaction analysis.
4.3 Functional analyses (II-III)

4.3.1 Computational analyses (II-III)

The identified mutations and variants were analysed for their putative effects on protein structure and function using the Polymorphism Phenotyping v2 tool (PolyPhen-2) (Adzhubei et al. 2010). The UniProt database accession numbers used for the protein sequence analyses were O75197 (LRP5), O94907 (DKK1) and P56704 (WNT3A). In addition, partial protein sequence alignments were assembled for all mutation sites: the LRP5 sequence alignment was created using the ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and WNT3A and DKK1 protein alignments using the NCBI HomoloGene system (http://www.ncbi.nlm.nih.gov/sites/entrez?db=homologene).

4.3.2 Constructs (II-III)

Wild type (WT) and mutant constructs of LRP5, DKK1 and WNT3A were applied in examining the effects of the mutations and variants on protein expression, activity of the canonical Wnt signalling pathway, and on transcription of Tph1 and 5-Htr1b genes in the case of LRP5 mutants. A list of the constructs is presented in Table 1. Mutant constructs were created using the QuikChange XL site-directed mutagenesis kit (Stratagene) with specifically designed primers, and the resulting construct sequences were confirmed by direct sequencing.
<table>
<thead>
<tr>
<th>Construct name</th>
<th>Description/Origin/Background</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-LRP5-mycHis</td>
<td>Wild type LRP5 containing mycHis tag in pcDNA3.1+p vector (II, II)</td>
</tr>
<tr>
<td>G171V-LRP5-mycHis</td>
<td>Mutant LRP5 created using in vitro mutagenesis (II)</td>
</tr>
<tr>
<td>C913fs-LRP5-mycHis</td>
<td>Mutant LRP5 created using in vitro mutagenesis (II)</td>
</tr>
<tr>
<td>R1036Q-LRP5-mycHis</td>
<td>Mutant LRP5 created using in vitro mutagenesis (II)</td>
</tr>
<tr>
<td>L1149Q-LRP5-mycHis</td>
<td>Mutant LRP5 created using in vitro mutagenesis (II)</td>
</tr>
<tr>
<td>G1185R-LRP5-mycHis</td>
<td>Mutant LRP5 created using in vitro mutagenesis (II)</td>
</tr>
<tr>
<td>WT-DKK1-flag</td>
<td>Wild type DKK1 containing flag tag pcDNA3.1+p vector (III)</td>
</tr>
<tr>
<td>R120L-DKK1-flag</td>
<td>Variant DKK1 created using in vitro mutagenesis (III)</td>
</tr>
<tr>
<td>WT-Wnt3a</td>
<td>Wild type Wnt3a in pIRES2-EGFP vector (III)</td>
</tr>
<tr>
<td>K51R-Wnt3a</td>
<td>Variant Wnt3a created using in vitro mutagenesis (III)</td>
</tr>
<tr>
<td>WT-Wnt1</td>
<td>Wild type Wnt1 in pcDNA3.1+ vector</td>
</tr>
<tr>
<td>STF</td>
<td>SuperTOPflash reporter construct (II, III)</td>
</tr>
<tr>
<td>β-Gal-CMV</td>
<td>β-galactosidase reporter construct (II, III)</td>
</tr>
<tr>
<td>pcDNA3.1+</td>
<td>Empty vector (Invitrogen) (II, III)</td>
</tr>
<tr>
<td>pIRES2-EGFP</td>
<td>Empty vector (Clontech) (III)</td>
</tr>
</tbody>
</table>

4.3.3 Expression and detection of LRP5 and DKK1 (II-III)

Chinese hamster ovarian (CHO) cells were used throughout the in vitro studies. The cells were cultured with 10% fetal bovine serum (FBS) (HyClone) in Dulbecco’s modified Eagle’s medium (DMEM) (BIOCHROM AG) and plated on ø10 cm plates at a density of 2×10⁶ cells/plate for transfection. Transfections were performed using 3 μg of DNA (WT- or mutant LRP5 or DKK1 construct) and Lipofectamine transfection reagent (Invitrogen) according to the manufacturer’s instructions. Cell media were collected and cells harvested using a 1% Triton-X-homogenisation buffer 48 h after the transfection.

SDS-PAGE gels were prepared for the analysis of LRP5 and DKK1 (7.5% and 10% SDS-PAGE gels, respectively). For electrophoreses, 25 μl of medium or cell lysate was mixed with 10 μl of SDS-PAGE loading buffer (20% SDS, 20% β-mercaptoethanol, 4× SDS-PAGE buffer), the sample was loaded on the gel and analysed under reducing conditions. LRP5 was detected in the Western blot using Anti-Myc tag, clone 9E10 antibody (Upstate) and DKK1 using AntiFLAG M2 antibody (Stratagene).
4.3.4 Producing Wnt3a- and DKK1-conditioned media (II-III)

Wnt3a-conditioned medium (Wnt3a-CM) was produced and collected from a commercial mouse cell line expressing Wnt3a (L Wnt3a; ATCC CRL-2647) following the instructions of the ATCC. A control-conditioned medium (L1-CM) was produced from a normal L1 cell line (ATCC CRL-2648), also according to the ATCC protocol. Transfection for producing wild type (WT-DKK1-CM) and variant (R120L-DKK1-CM) DKK1 conditioned media is described in Methods 4.3.3. DKK1 conditioned media were collected from the plates 48 h after transfections and the presence of DKK1 was verified using a Western blot.

4.3.5 Luciferase gene reporter assay (II-III)

A Luciferase assay method was applied to study the effects of LRP5 mutants and DKK1 and WNT3A variants on the activity of canonical Wnt signalling pathway. CHO cells were plated onto a 24-well plate at a density of 2×10⁴ cells/well 24 h prior to transfection. For studying the differences between WT-LRP5 and mutant LRP5s, transfection complexes included 20 ng of WT- or mutant LRP5 construct, 100 ng of STF and 5 ng of β-Gal-CMV. The total amount of DNA was made up to 250 ng/well by the addition of pcDNA3.1+ vector to the complex. After five hours of incubation, 500 μl of medium was added onto the cells. The type of medium depended on the study set-up and the medium compositions used were as follows: I) 500 μl of 10% FBS-DMEM, II) 300 μl of either Wnt3a-CM or control L1-CM and 200 μl of 10% FBS-DMEM, or III) 300 μl of WT-DKK1-CM or R120L-DKK1-CM and 200 μl of 10% FBS-DMEM. The cells were lysed and collected 48 h later.

The studies of Wnt3a and Wnt1 were carried out using procedures similar to those of the LRP5 studies except for the composition of the transfection complexes. When comparing signalling activities of Wnt3a or -1, the transfection complexes consisted of 20 ng of WT-LRP5 or mutant LRP5, 10 ng of WT-Wnt3a or Wnt1, 100 ng of STF and 5 ng of β-Gal-CMV. When the WT-Wnt3a was compared to K51R-WNT3A the transfection complexes consisted of 20 ng of WT-LRP5, 10 ng of WT-Wnt3a or K51R-Wnt3a, 100 ng of STF and 5 ng of β-Gal-CMV. In both studies the total amount of DNA was brought to 250 ng by the addition of 65 ng of pcDNA3.1+. Five hours later 500 μl of 10%FBS-DMEM was added onto the cells and cells were harvested after 48 hours. All transfections were performed in triplicate and repeated at least three separate times.
4.3.6 Measuring luciferase activity (II-III)

Transfected cells were lysed with cell culture lysis reagent (CCLR) according to Promega instructions. A solution of 10 μl of cell lysate and 50 μl of Luciferase Assay Reagent (Promega) was prepared for measurement of luciferase activity (STF). β-galactosidase activity was determined from a solution of 10 μl of cell lysate and 70 μl of 1×CPRG (chlorophenol red β-D-galactopyranoside) substrate (Roche Diagnostics). Both measurements were recorded using a Victor3™ V 1420 Multilabel Counter (Perkin Elmer), and the results were given as relative luciferase units (RLU) calculated by dividing the luciferase activity by the β-galactosidase activity.

4.3.7 Quantitative-PCR (II)

Quantitative real-time PCR (qPCR) was performed to examine the effect of LRP5 mutants on Tph1 and 5-Htr1b expression. CHO cells were plated on 6-well plates at a density of 1×10⁵ cells/well and transfections were performed 24 h later. Lipofectamine (Invitrogen) transfection reagent was used with 2 μl of WT-LRP5, mutant LRP5 or pcDNA3.1+. After four hours, either 1000 μl of 10% FBS-DMEM, 600 μl Wnt3a-CM and 400 μl of 10% FBS-DMEM or 600 μl of control L1-CM and 400 μl of 10% FBS-DMEM were added to the wells and cells were harvested 48 h later. All experiments were performed in triplicate and repeated on at least three separate occasions.

Total RNA was isolated using the E.Z.N.A. RNA isolation kit with treatment of RNase-free DNase (OMEGA Bio-Tek), and RNA concentrations were determined using a NanoDrop™ ND-100 spectrophotometer (Thermo Scientific). cDNA was synthesised by reverse transcript PCR (RT-PCR) from 1 μg of RNA using an iScript™ cDNA Synthesis kit (BioRad). qPCR primers were designed based on murine sequences for Tph1, 5-Htr1b and β-actin, which was used as a standard control for the analysis. The iTaq™ SYBR Green Supermix with ROX (BioRad) was used for qPCR amplification. qPCR reactions were performed in a Mx3005P QPCR instrument (Stratagene), and the data were computed using MxPro – Mx3005P v4.10 software (Stratagene). The change in gene expression upon treatment was given as relative expression (fold relative to the non-treated control, i.e. pcDNA3.1+ transfected sample) after normalising to β-actin, and calculated by the 2−ΔΔCt method (Livak & Schmittgen 2001).
4.4 Statistical methods (I-III)

In article I, data were tested for potential deviation from Hardy-Weinberg equilibrium using the chi-square test. The likelihood ratio test was applied for disease association studies on alleles and genotypes. Variables differing between the cases and controls and associated with the polymorphisms were included as co-variables in multivariate analyses. Dominant, additive, recessive, and general genetic models were defined and tested for all the polymorphisms. Haplotype frequencies and pair-wise linkage disequilibrium (D') and correlation coefficient ($r^2$) values were established using Haploview software (MIT/Harvard Broad Institute Cambridge, MA) (Barrett et al. 2005). The haplotypes were reconstructed statistically from population genotype data using the PHASE program with the Markov chain method for haplotype assignment (Stephens et al. 2001). Potential risk or protective haplotypes were identified by comparing haplotype frequencies between the cases and controls using Fisher’s exact probability test or the chi-square test. The robustness of the associations was evaluated with a permutation test (100 permutations).

Crude and adjusted odds ratios and their 95% confidence intervals (CIs) were calculated using the SPSS statistical package (SPSS, Chicago, IL), and interactions between the polymorphisms were investigated by stratification and logistic regression analysis. The statistical significance of a p-value was defined as the 5% level.

For the in vitro studies of non-OI primary osteoporosis mutants in articles II and III, the statistical analyses were performed using the Student’s t test. Values of < 0.05 were considered statistically significant, and the results were presented as mean±standard deviation (SD).
5 Results

5.1 Genetic factors in femoral neck stress fractures (I)

The association analyses in this study revealed specific haplotypes and haplotype interactions, which increased the risk for femoral neck stress fractures via reduced weight and BMI. New sequence variants or mutations were not found in the study.

5.1.1 Clinical findings (I)

The femoral neck stress fracture patients were smaller in size than the controls as the two groups differed from each other by height ($p = 0.006$), weight ($p = 0.0005$) and BMI (Table 2). The control group differed from the cases in regard to age. No statistically significant association was detected between age and BMI or BMI and smoking between control group and cases (data not shown).

Since the control group data were collected at a later time than those for the cases, the clinical parameters of cases were also compared to those of their healthy contemporaries. This was done to rule out the possibility that the time discrepancy was affecting conscripts’ sizes. The same result was obtained from these analyses: the cases had lower body weight and BMI than their healthy contemporaries, showing that the differences in parameters were not due to the temporal discrepancy between the two original groups (Table 2). Unfortunately, DNA was not available from the contemporaries hence genetic analyses could not be performed. Cases with low body weight or BMI had a significantly higher risk for femoral neck stress fractures.
Table 2. Comparison of characteristics of femoral neck stress fracture cases, controls and contemporaries of cases.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases</th>
<th>Controls</th>
<th>p-value</th>
<th>Contemporaries</th>
<th>p-value</th>
<th>p-value controls vs. contemporaries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>20.3 ± 1.6</td>
<td>18.9 ± 0.5</td>
<td>0.0005</td>
<td>20.4 ± 1.5</td>
<td>0.7997</td>
<td>0.0005</td>
</tr>
<tr>
<td>Height</td>
<td>177.1 ± 6.0</td>
<td>179.6 ± 6.2</td>
<td>0.006</td>
<td>179.3 ± 5.6</td>
<td>0.0098</td>
<td>0.1246</td>
</tr>
<tr>
<td>Weight</td>
<td>68.9 ± 9.6</td>
<td>77.3 ± 13.1</td>
<td>0.0005</td>
<td>75.5 ± 10.0</td>
<td>0.00001</td>
<td>0.2201</td>
</tr>
<tr>
<td>BMI</td>
<td>22.0 ± 2.9</td>
<td>23.9 ± 3.5</td>
<td>0.0005</td>
<td>23.5 ± 2.8</td>
<td>0.0004</td>
<td>0.2627</td>
</tr>
<tr>
<td>Smoking*</td>
<td>29 (42.0%)</td>
<td>67 (56.8%)</td>
<td>0.069</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

*N and % of smokers; n.a. = not analysed

5.1.2 Candidate gene analysis (I)

Five genes associated with risk for low bone mass or osteoporotic fractures were screened for disease-causing mutations in 72 femoral neck stress fracture patients and 120 healthy controls. The genes included COL1A1, COL1A2, LRP5, OPG and ESR1, and the coding regions and their boundaries were examined using CSGE (Gong et al. 2001, Körkkö et al. 1998, Simonet et al. 1997, Uitterlinden et al. 1998). Previously reported sequence variations were detected in all candidate genes both in cases and controls (data not shown), but no novel variations were observed as verified from the NCBI GenBank.

5.1.3 Association analysis of SNPs (I)

Fifteen SNPs in 7 genes (COL1A1, COL1A2, CTR, IL-6, OPG, VDR and LRP5) were genotyped to test for possible allelic associations. Genotype frequencies for all SNPs were in Hardy-Weinberg equilibrium. The resulting allele frequencies were compared between cases and controls and are shown in Table 2 in article I. The frequency of the LRP5 rs2277268 minor allele A was marginally elevated in the cases and associated significantly with low body weight (p = 0.036) and low BMI (p = 0.017). The LRP5 rs4988321 minor allele A also associated with lower height (p = 0.025), whereas the VDR BsmI minor allele A associated with higher BMI (p = 0.04). Only BMI was included as a covariate in the subsequent analyses, as neither of the polymorphisms associated with age or smoking.

The COL1A1 rs2586488 and COL1A2 rs3216902 SNPs associated with stress fractures under a recessive model (Table 3), and the risk was almost three-fold in
subjects carrying the *LRP5* rs2277268 minor allele as compared to non-carriers (Table 3). However, the associations were lost after adjusting for BMI. SNPs on *VDR*, *CTR* and *IL-6* did not significantly associate with stress fractures (Table 3).

**Table 3.** Genotype distributions, odds ratios (OR), and their 95% confidence intervals (CI) and p-values for the genetic models. Statistically significant values are marked in italics.

<table>
<thead>
<tr>
<th>Gene/ SNP</th>
<th>Genotype</th>
<th>Controls/ Cases</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDR</td>
<td>rs10735810</td>
<td>C/C</td>
<td>41/29</td>
<td>1</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/C</td>
<td>58/30</td>
<td>0.71</td>
<td>0.38-1.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/T</td>
<td>11/10</td>
<td>1.50</td>
<td>0.38-1.35</td>
</tr>
<tr>
<td></td>
<td>rs1544410</td>
<td>G/G</td>
<td>46/17</td>
<td>1</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G/A</td>
<td>58/25</td>
<td>0.54</td>
<td>0.28-1.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/A</td>
<td>16/10</td>
<td>0.78</td>
<td>0.32-1.91</td>
</tr>
<tr>
<td></td>
<td>rs731236</td>
<td>T/T</td>
<td>49/37</td>
<td>1</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/C</td>
<td>54/25</td>
<td>0.61</td>
<td>0.32-1.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C/C</td>
<td>17/10</td>
<td>0.78</td>
<td>0.32-1.90</td>
</tr>
<tr>
<td>IL6</td>
<td>rs1800795</td>
<td>G/G</td>
<td>35/15</td>
<td>1</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G/C</td>
<td>56/42</td>
<td>1.75</td>
<td>0.85-3.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C/C</td>
<td>29/15</td>
<td>1.21</td>
<td>0.51-2.88</td>
</tr>
<tr>
<td>CTR</td>
<td>rs1922295</td>
<td>T/T</td>
<td>59/45</td>
<td>1</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/C</td>
<td>49/19</td>
<td>0.51</td>
<td>0.25-0.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C/C</td>
<td>12/8</td>
<td>0.87</td>
<td>0.33-2.32</td>
</tr>
<tr>
<td>COL1A1</td>
<td>rs1800012</td>
<td>G/G</td>
<td>85/53</td>
<td>1</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G/T</td>
<td>34/17</td>
<td>0.80</td>
<td>0.41-1.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/T</td>
<td>1/2</td>
<td>3.21</td>
<td>0.28-3.25</td>
</tr>
<tr>
<td></td>
<td>rs2696247</td>
<td>T/T</td>
<td>189/100</td>
<td>1</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/C</td>
<td>123/88</td>
<td>1.27</td>
<td>0.68-2.35</td>
</tr>
<tr>
<td></td>
<td>rs2586488</td>
<td>C/T</td>
<td>58/27</td>
<td>0.78</td>
<td>0.41-1.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/T</td>
<td>12/15</td>
<td>2.08</td>
<td>0.86-5.04</td>
</tr>
<tr>
<td>COL1A2</td>
<td>rs406226</td>
<td>A/A</td>
<td>86/50</td>
<td>1</td>
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<td>A/G</td>
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<td></td>
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<td>G/G</td>
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67
Gene/SNP | Genotype | Controls/ Cases | OR | 95% CI | p-value |
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<td>Dominant</td>
<td>Additive</td>
<td>Recessive</td>
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<tr>
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<td>G/A</td>
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<tr>
<td></td>
<td>A/A</td>
<td>0/2</td>
<td>-</td>
<td>-</td>
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<tr>
<td>rs3216092</td>
<td>Ins/Ins</td>
<td>52/29</td>
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<td>0.15-1.25</td>
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</tr>
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<td></td>
<td>G/A</td>
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<td>2.72</td>
<td>1.10-6.73</td>
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<tr>
<td></td>
<td>G/A</td>
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<td>A/G</td>
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<tr>
<td></td>
<td>C/T</td>
<td>13/100</td>
<td>1.33</td>
<td>0.55-3.21</td>
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</tr>
</tbody>
</table>

* OR = 2.37, 95% CI 1.04-5.6; <sup>b</sup> OR = 0.35, 95% CI 0.13-0.98. Body mass index (BMI) was used as covariate in the analyses.

5.1.4 Linkage disequilibrium, haplotype and interaction analyses (I)

Pairwise linkage disequilibrium was examined between three SNPs within **COL1A1**, **COL1A2** and **VDR** and four SNPs within **LRP5**. The results for linkage disequilibrium (D') are presented in D’ plots in Figures 1A-D in article I.

The SNPs within **COL1A1** and **COL1A2** were in linkage disequilibrium (Figure 1 in article I). Eight haplotypes were observed in **COL1A2**, three of which were present in both cases and controls and four were found only in controls. The **COL1A2** haplotype frequencies differed marginally between cases and controls (p = 0.0197). No significant association was observed between femoral neck stress fractures and **COL1A2** haplotypes. **VDR** SNPs FokI and TaqI-BsmI located at separate haplotype blocks and did not associate with stress fractures.

Of the seven haplotypes observed in **LRP5** the A-G-G-C haplotype was more frequent in cases than in controls (p = 0.031). It increased the risk of stress fractures as compared to non-carriers (OR 2.72: 95% CI 1.10–6.37, p = 0.031), and also the risk of low BMI (OR 2.50; 95% CI 1.03–6.07, p = 0.04). The statistical significance of the association was lost after adjusting for BMI (OR
2.04; 95% CI 0.79–5.22), indicating that BMI mediates the association for stress fractures.

Haplotype interactions between the *CTR-VDR, VDR-LRP5* and *CTR-VDR-LRP5* genes were analysed. The combination of the *LRP5* haplotype A-G-G-C and the *VDR* haplotype C-A was found to increase the risk for stress fractures by 3.85-fold (95% CI 1.16–12.84) as compared to the carriers of only the C-A haplotype (Table 4). In subjects without the C-A haplotype the risk was marginally increased (1.78; 95% CI 0.96–3.30) (Table 4). The association of these joined haplotypes with femoral neck stress fractures was mediated at least partly by BMI, for the associations were lost after adjustment for BMI (OR 3.10; 95% CI 0.87–11.1 for the joint effect of haplotypes). The most important finding, however, was the interaction between a *CTR* minor allele C and the *VDR* haplotype C-A, revealing an increased risk of fracture in subjects without the C allele or the C-A haplotype or without either one (OR = 3.22; 95% CI 1.38–7.49, p = 0.007).

<table>
<thead>
<tr>
<th>LRP5 A-G-G-C</th>
<th>VDR C-A</th>
<th>Controls N (fr.)</th>
<th>Cases N (fr.)</th>
<th>OR (95% CI)</th>
<th>p-value</th>
<th>Adjusted OR 1</th>
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<tr>
<td>- or +</td>
<td>-</td>
<td>50 (0.383)</td>
<td>37 (0.444)</td>
<td>1.78 (0.96–3.30)</td>
<td>0.07</td>
<td>1.85 (0.96–3.55)</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>65 (0.542)</td>
<td>27 (0.375)</td>
<td>1.00</td>
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<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>5 (0.042)</td>
<td>8 (0.111)</td>
<td>3.85 (1.16–12.84)</td>
<td>0.028</td>
<td>3.10 (0.87–11.1)</td>
</tr>
</tbody>
</table>

Table 4. *LRP5* A-G-G-C haplotype and *VDR* C-A haplotype interaction. Statistically significant values are marked in italics.

1 OR after adjustment for body mass index (BMI)

5.2 Studying the components of the canonical Wnt signalling pathway in non-OI primary osteoporosis (II-III, unpublished results)

Out of the nine candidate genes studied, three were found with putative disease associating variants, namely the genes encoding for LRP5, WNT3A and DKK1. Furthermore, the *in vitro* studies gave suggestive results for a mechanism of action for some of these variants.
5.2.1 Clinical features (II-III)

Patients were diagnosed with non-OI primary osteoporosis based on the criteria described in the methods section (Gordon et al. 2008, Ward LM 2003). The clinical data for the non-OI primary osteoporosis patients are presented in Table 5. Unfortunately, clinical information was not available for three patients (M15, M17 and M20). The clinical data on patients M11, M13, OP4 and OP17, who were found with mutations, are described in detail in chapter 5.2.2. The number of peripheral fractures in patients varied from zero to nine, the average rate being between two and three (the average was calculated based on numeric data available). Peripheral fractures were not observed in five patients. Vertebral compression fractures were encountered in 16 patients, and their severity ranged from mild (in six patients) to severe (five patients). Ten patients had not sustained any vertebral fractures and vertebral fractures were not assessed for three patients. In one patient the Z score value was >-1.0, in seven patients the value was <-1.5, and in 20 patients the value exceeded the diagnostic threshold value of osteoporosis (< -2.0).

Table 5. Clinical data for the non-OI primary osteoporosis patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age of onset</th>
<th>BMD (Z score)</th>
<th>ALP (Z score)</th>
<th>Peripheral fractures (N)</th>
<th>Compression fractures¹</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>M1</td>
<td>M</td>
<td>12</td>
<td>-3.2</td>
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<td>0</td>
<td>Mild</td>
</tr>
<tr>
<td>M2</td>
<td>M</td>
<td>11</td>
<td>-2.2</td>
<td>NA</td>
<td>2</td>
<td>N</td>
</tr>
<tr>
<td>M3</td>
<td>F</td>
<td>12</td>
<td>-2.1</td>
<td>NA</td>
<td>1</td>
<td>N</td>
</tr>
<tr>
<td>M4</td>
<td>M</td>
<td>13</td>
<td>-4</td>
<td>NA</td>
<td>3</td>
<td>Mild</td>
</tr>
<tr>
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<td>8</td>
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<td>N</td>
</tr>
<tr>
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<td>F</td>
<td>6</td>
<td>-2</td>
<td>NA</td>
<td>1</td>
<td>N</td>
</tr>
<tr>
<td>M7</td>
<td>F</td>
<td>14</td>
<td>-2.1</td>
<td>NA</td>
<td>1</td>
<td>N</td>
</tr>
<tr>
<td>M8</td>
<td>M</td>
<td>10</td>
<td>-2.2</td>
<td>NA</td>
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<td>N</td>
</tr>
<tr>
<td>M9</td>
<td>M</td>
<td>2.5</td>
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<td>NA⁴</td>
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<td>Multiple</td>
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<td>M</td>
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<td>NA⁵</td>
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<td>Yes</td>
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<tr>
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<td>M</td>
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<td>-2.0</td>
<td>0.5</td>
<td>5</td>
<td>N</td>
</tr>
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<tr>
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<td>9</td>
<td>N</td>
</tr>
<tr>
<td>M19</td>
<td>M</td>
<td>8</td>
<td>NA⁷</td>
<td>NA¹</td>
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<td>4</td>
</tr>
<tr>
<td>Set II</td>
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</tr>
<tr>
<td>OP1</td>
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<td>-1.5</td>
<td>6</td>
<td>Moderate</td>
</tr>
<tr>
<td>OP2</td>
<td>M</td>
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<td>-1.0</td>
<td>-1.8</td>
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</tr>
<tr>
<td>OP3</td>
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<td>6</td>
<td>1.0</td>
<td>-1.4</td>
<td>4</td>
<td>NA</td>
</tr>
<tr>
<td>Patient</td>
<td>Sex</td>
<td>Age of onset</td>
<td>BMD (Z score)</td>
<td>ALP (Z score)</td>
<td>Peripheral fractures (N)</td>
<td>Compression fractures</td>
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<td>--------------</td>
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<td>--------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>OP6</td>
<td>F</td>
<td>4</td>
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<td>-1.1</td>
<td>8</td>
<td>Severe</td>
</tr>
<tr>
<td>OP7</td>
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<td>14</td>
<td>-2.6</td>
<td>-0.1</td>
<td>3</td>
<td>N</td>
</tr>
<tr>
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<td>13</td>
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<td>-0.8</td>
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</tr>
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<td>-1.7</td>
<td>4</td>
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</tr>
<tr>
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<td>10</td>
<td>-2.4</td>
<td>-1.8</td>
<td>&gt;8</td>
<td>Severe</td>
</tr>
</tbody>
</table>

1 Mild, compression fractures consists of end-plate deformities; moderate, mild anterior wedging; severe, severe anterior wedging and/or reduced posterior height of the vertebra; N none; NA, not assessed; a 249 (15-350) U/l; b 0.41 gm/cm² at proximal radius and 0.25 gm/cm² at distal radius; c 357 (15-350) U/l; d 112 (15-350) U/l; e 0.49 g/m²; f 156 (15-350) U/l.

### 5.2.2 Identification of mutations associated with non-OI primary osteoporosis (II-III)

Previously identified sequence variations were observed from all candidate genes and are presented in Table 6. Altogether two new mutations (in LRP5) and two variants (one in DKK1 and one in WNT3A) were identified from four non-OI primary osteoporosis patients. From the families of these patients 11 affected and 11 unaffected family members were recruited in the genetic analyses. No DNA was available for two family members. The variants were observed in six affected family members (Figure 6) described in detail below and none of the variants was observed in controls. However, p.K51R has been reported in the NCBI database (http://www.ncbi.nlm.nih.gov/snp/), as a part of exome sequencing project with a rare occurrence of 1/4522 (rs145797401). In addition, a rare variant in the DKK1 promoter was detected, but it did not associate with the bone phenotype.

Patient M11 (Figure 6A II:1) had recurrent fractures and repeatedly reduced BMD measures during early childhood and adolescence, and was diagnosed with non-OI primary osteoporosis. The patient’s BMD values had normalised after he reached adult age (34 years), except for the osteopenic T-score -1.5 in the femoral neck. Patient M11 was found with a missense mutation c.3446T>A, p. L1149Q in the LRP5 gene. The mutation was detected also in the patient’s mother and sister.
(Figure 5A I:1 and II:2, respectively), who were both affected with osteoporosis. The BMD T-score values for the mother’s lumbar spine and femoral neck at 48 years were -2.3 and -1.3, respectively, and for the sister the BMD T-scores at same sites at 25 years were -1.4 and -2.5. The healthy father (Figure 6A I:2) did not have the mutation.

Patient M13 (Figure 6B II:1) had his first fracture at 7 years and by the age of 14.5 years he had sustained 8 peripheral fractures. He was first presented at the age of 13 and at that time his biochemical values and renal function were normal. No compression fractures or structural abnormalities were observed, however his radiographs showed signs of reduced mineralisation. The total and trabecular volumetric BMD Z-scores were reduced (-1.5 and -2.8, respectively) determined with peripheral quantitative computed tomography (pQCT; Stratec QCT 900). DXA measurements showed a lumbar spine BMD Z-score -2.4. Finally, bone biopsy revealed reduced bone volume and loss of trabecular connectivity, consistent with osteoporosis. M13 had a $LRP5$ missense mutation c.3553G>A, p.G1185R. The father (Figure 6B I:2) had the same mutation and his BMD measurements on DXA were slightly reduced, the T-scores being -0.3 at lumbar spine and -1.7 at femoral neck. The unaffected mother and sister of M13 (Figure 6B I:1 and II:2, respectively) did not carry the mutation and, apart from the father, there is no previous history of osteoporosis in the family.

Patient OP4 (Figure 6C III:1) had sustained three fractures, two of which resulted from minimal trauma. The first fracture occurred in the right forearm at 6 years, the second one in the right wrist and the third fracture in the hip at 15 years (2001), followed by investigations for osteoporosis. Biochemical results were found normal and DXA measurements revealed a lumbar spine (L1-4) BMD of 0.631 g/cm$^2$, which was equivalent to a Z-score of -4.8. In the beginning, OP4 was treated with calcium and vitamin D, and peroral alendronate treatment was used between January and November 2003. The diagnosis of non-OI primary osteoporosis was confirmed from a bone biopsy taken in 2004. OP4 had a missense mutation c.152A>G, p.K51R in $WNT3A$. The patient’s affected mother and sister (Figure 6C II:1 and III:2, respectively) shared the mutation, while the healthy siblings (Figure 6C III:3 and III:4) and father (Figure 6C II:2) did not. The mother’s BMD had decreased after menopause and the sister had sustained two fractures resulting from moderate impact traumas. There was also a history of osteoporosis on the paternal side of the family and the father himself had had sport related fractures.
Patient OP17 (Figure 6D II:1) had first fractured his collar bone at 5 years, and T5 and T7 vertebrae at 10 years following low impact traumas. The first measurements of BMD at 10 years showed a mild reduction at the lumbar spine (L1-L4), the rate being 0.564 g/cm² and corresponding to a Z-score of -1.9. During the years 2002–2005 his alkaline phosphatase levels had been slightly elevated from 192 to 434 IU/l. The diagnosis of non-OI primary osteoporosis was confirmed from bone biopsies and pamidronate treatment was started. OP17 had a DKK1 missense mutation c.359G>T, p.R120L, which was also observed in his affected sister. Both parents were affected: the father (Figure 6D I:2) did not have the mutation, whereas the mother (Figure 6D I:1) declined the genetic analysis. The affected sister (Figure 6D II:2) had osteoporosis as indicated by a Z-score of -2.1 and has had vertebral compression fractures. The mother was osteopenic with a Z-score of -1 and the father had osteoporosis with a Z-score of -2.7.
Fig. 6. Pedigrees of A) patient M11 (II:1) with *LRP5* mutation L1149Q, B) patient M13 (II:1) with *LRP5* mutation G1185R, C) patient OP4 (III:1) with *WNT3A* variant K51R, D) patient OP17 (II:1) with *DKK1* variant R120L. Explanations for the symbols are presented in the box within the schematic.
Table 6. SNPs observed in the candidate genes analysed.

<table>
<thead>
<tr>
<th>Candidate gene</th>
<th>GenBank rs-number</th>
<th>Nucleotide change</th>
<th>Predicted consequence</th>
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<td>g.5109_5135del27ins(CTG)10</td>
<td>L15_L20del</td>
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<td>rs545382</td>
<td>g.95906T&gt;C</td>
<td>F549F</td>
</tr>
<tr>
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<td>rs2277268</td>
<td>g.99015G&gt;A</td>
<td>E644E</td>
</tr>
<tr>
<td></td>
<td>rs498321</td>
<td>g.99082G&gt;A</td>
<td>V667M</td>
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<td>rs2306862</td>
<td>g.102403C&gt;T</td>
<td>N740N</td>
</tr>
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<td></td>
<td>rs4988322</td>
<td>g.102507T&gt;C</td>
<td>Splice site</td>
</tr>
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<td></td>
<td>rs556442</td>
<td>g.117583G&gt;A</td>
<td>V1119V</td>
</tr>
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<td>rs3736228</td>
<td>g.126188C&gt;T</td>
<td>A1330V</td>
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<td>G96R</td>
</tr>
<tr>
<td>WNT10B</td>
<td>rs1051886</td>
<td>g.10653C&gt;T</td>
<td>H353H</td>
</tr>
<tr>
<td>SOST</td>
<td>rs17882143</td>
<td>g.5075G&gt;A</td>
<td>V10i</td>
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<tr>
<td>AXIN1</td>
<td>rs1805105</td>
<td>g.11201T&gt;C</td>
<td>D254D</td>
</tr>
<tr>
<td></td>
<td>rs2301522</td>
<td>g.47512T&gt;C</td>
<td>Intrinsic</td>
</tr>
<tr>
<td></td>
<td>rs62032861</td>
<td>g.53178G&gt;A</td>
<td>Intrinsic</td>
</tr>
<tr>
<td></td>
<td>rs214250</td>
<td>g.59243G&gt;A</td>
<td>S428S</td>
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<tr>
<td></td>
<td>rs214252</td>
<td>g.60281T&gt;C</td>
<td>A609A</td>
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<tr>
<td></td>
<td>rs118063900</td>
<td>g.66283C&gt;T</td>
<td>Splice site</td>
</tr>
<tr>
<td></td>
<td>rs34015754</td>
<td>g.69276G&gt;A</td>
<td>R840Q</td>
</tr>
<tr>
<td>TPH1</td>
<td>rs1799913</td>
<td>g.20061C&gt;A</td>
<td>Splice site</td>
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<tr>
<td></td>
<td>rs211101</td>
<td>g.23032G&gt;A</td>
<td>Intrinsic</td>
</tr>
<tr>
<td>5-HTR1B</td>
<td>rs6297</td>
<td>g.78172092C&gt;T</td>
<td>S43S</td>
</tr>
<tr>
<td></td>
<td>rs6296</td>
<td>g.78162260C&gt;G</td>
<td>V287V</td>
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Predisposing variants for non-OI primary osteoporosis were detected on three candidate genes encoding for \textit{LRP5}, \textit{DKK1} and \textit{WNT3A}. All variants led to amino acid substitutions. The functional effect of variants on protein structure or function was studied using computational programs PolyPhen-2 tool (Adzhubei \textit{et al.} 2010), MUpro (Cheng \textit{et al.} 2006), I-Mutant2.0 (Capriotti \textit{et al.} 2005), SIFT (Kumar \textit{et al.} 2009, Ng & Henikoff 2001, Ng & Henikoff 2002, Ng & Henikoff 2003) and SNAP (Bromberg & Rost 2007), and the results are presented in Table 7.

<table>
<thead>
<tr>
<th>Gene / variant</th>
<th>PolyPhen-2</th>
<th>MUpro</th>
<th>I-Mutant2.0</th>
<th>SIFT</th>
<th>SNAP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DKK1 / R120L</strong></td>
<td>Probably damaging (score 0.996, sensitivity 0.46, specificity 0.97)</td>
<td>Method 1: decrease stability (CS:0.51303469)</td>
<td>Tolerated (RI:5)</td>
<td>Non-neutral (RI:3, expected accuracy 78%)</td>
<td></td>
</tr>
<tr>
<td><strong>WNT3A / K51R</strong></td>
<td>Benign (score 0.163, sensitivity 0.91, specificity 0.83)</td>
<td>Method 1: increase stability (CS:0.16764826)</td>
<td>Decrease (RI:5)</td>
<td>Neutral (RI:6, expected accuracy 92%)</td>
<td></td>
</tr>
<tr>
<td><strong>LRP5 / G171V</strong></td>
<td>Probably damaging (score 1.00, sensitivity 0, specificity 1.9)</td>
<td>Method 1: decrease stability (CS: -0.26061539)</td>
<td>Decrease (RI:2) Affects protein function (score 0.01, median sequence conservation 3.06, no sequences 14)</td>
<td>Non-neutral (RI:4, expected accuracy 82%)</td>
<td></td>
</tr>
<tr>
<td>Gene / variant</td>
<td>PolyPhen-2</td>
<td>MUpro</td>
<td>I-Mutant2.0</td>
<td>SIFT</td>
<td>SNAP</td>
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</tr>
<tr>
<td>LR5p/C913fs</td>
<td>Possibly damaging</td>
<td>Method 1: increase stability (CS:0.17731375)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LR5p/R1036Q</td>
<td>Possibly damaging</td>
<td>Method 1: increase stability (CS:0.23560379)</td>
<td>Decrease (RI:7) Tolerated</td>
<td>(score 0.21, median sequence conservation: 3.06, no sequences 14)</td>
<td>Non-neutral (RI:3, expected accuracy 78%)</td>
</tr>
<tr>
<td>LR5p/L1149Q</td>
<td>Probably damaging</td>
<td>Method 1: decrease stability (CS:-1)</td>
<td>Decrease (RI:9) Affects protein function</td>
<td>(score 0.01, median sequence conservation: 3.06, no sequences 14)</td>
<td>Non-neutral (RI:4, expected accuracy 82%)</td>
</tr>
<tr>
<td>LR5p/G1185R</td>
<td>Possibly damaging</td>
<td>Method 1: decrease stability (CS:-0.77771829)</td>
<td>Decrease (RI:8) Tolerated</td>
<td>(score 0.37, median sequence conservation: 3.06, no sequences 14)</td>
<td>Non-neutral (RI:3, expected accuracy 78%)</td>
</tr>
</tbody>
</table>

All the identified variants were located in coding regions of the genes, and at conserved amino acids, as demonstrated by the interspecific sequence alignments (Figure 7). The novel LR5p mutations (L1149Q and G1185R) are situated at the fourth YWTD/EGF domain of LR5p. The WNT3A variant is located 79 nucleotides downstream from an important palmitoylation site (cysteine) and 106 nucleotides apart from a N-linked glycosylation site (Doubravska et al. 2011, Nusse 2003). The modifications on these sites are important for Wnt secretion and activity (Doubravska et al. 2011, Nusse 2003).
Fig. 7. A) partial alignment of the human LRP5 protein sequence (NP_002326.2) with Pan troglodytes (XP_508605.2), Bos Taurus (XP_614220.3), Mus musculus (NP_032539.1), Rattus norvegicus (XP_215187.3), Gallus gallus (NP_001012915.1), Danio rerio (XP_696943.4), Drosophila melanogaster (XP_320740.4), B) partial alignment of the human WNT3A protein sequence (NP_149122.1) with its equivalents in Canis lupus familiaris (XP_539327.2), Bos Taurus (XP_606943.4), Drosophila melanogaster (NP_524737.2) and Anopheles gambiae (XP_320740.4), C) Partial protein sequence alignment of the human DKK1 sequence (NP_036374.1) with its equivalents in Pan troglodytes (XP_001163253.1), Bos Taurus (XP_580572.2), Mus musculus (NP_034181.2), Rattus norvegicus (XP_219804.3), Gallus gallus (XP_571078.1). Numbers within sequences depict the number of extra amino acids as compared to Homo sapiens sequence (these are marked with spaces in Homo sapiens and other sequences), lines show lacking sequence, and the arrowheads below the alignments point to sequence variations.

The significance of the LRP5 mutations and DKK1 variant on protein expression and activity of canonical Wnt signalling were assessed in in vitro assays. Also the effects of the LRP5 mutants on the expression of Tph1 and 5-Htr1b were examined. The expression levels of LRP5 mutants and DKK1 variant were first studied and compared to those of wild type constructs using SDS-PAGE and Western blots. No differences were observed between the expression of the WT-
LRP5 and the variant forms (G171V, C913fs, R1036Q, L1149Q and G1185R); and the expression of R120L-DKK1 did not differ from that of WT-DKK1.

Fig. 8. Luciferase activity in CHO cells co-transfected with WT-LRP5 or mutant LRP5, STF and β-galactosidase in the presence of Wnt3a-CM (white bars) or L1 control medium (grey bars). Activities of the pathway are presented in relative luciferase units (RLU) determined by the ratio between the luciferase and β-galactosidase activities and given as a fold change relative to the corresponding samples treated with 10% FBS-DMEM. * p < 0.05 and ** p < 0.01 as compared with WT-LRP5. Results are expressed as means ± SD.

The luciferase assay method was applied to study the effects of LRP5 mutations, and DKK1 and WNT3A variants on the activity of the canonical Wnt signalling pathway. The LRP5 constructs were transfected into CHO cells, and studies were carried out using one of three media: 10% FBS-DMEM, Wnt3a-CM or L1-CM. The results of Wnt3a-CM treated cells were reported as fold changes relative to results obtained from 10%FBS-DMEM treated cells. As an activator of the pathway, Wnt3a increased the activity in all LRP5 constructs ranging between 4 (C913fs) to 9-fold (G171V) when compared to the corresponding samples treated with 10%FBS-DMEM (Figure 8). In addition, Wnt3a elucidated the signalling differences between the LRP5 constructs. When the signalling activities of mutants were compared to that of WT-LRP5, the activity of the HBM mutant G171V resembled that of WT-LRP5, whereas a decreasing trend in activity was
observed in mutants C913fs (p = 0.0023), R1036Q (p = 0.084) and L1149Q (p = 0.043). The activity in LRP5 G1185R was similar to that of WT-LRP5.

Parallel results were obtained in our yet unpublished work, where CHO cells were co-transfected with Wnt1 and LRP5 constructs. The results were largely congruent with those of the Wnt3a-CM studies, proving a significant decrease in signalling activity in the LRP5 mutants C913fs (p < 0.0001) and L1149Q (p < 0.0001) (Figure 9). Of note is that the signalling activity of the HBM mutation G171V was not increased in Wnt1 co-transfected cells, but was slightly decreased, yet still remained close to that of WT-LRP5. These results suggest that the LRP5 mutants affect the interactions with two Wnt ligands similarly (unpublished results).

The in vitro studies of signalling activities of WT- and K51R-Wnt3a showed a statistically significant reduction in K51R-Wnt3a signalling activity as compared to WT-Wnt3a (p = 0.01, Figure 10). The DKK1 variant R120L-DKK1 and the WT-DKK1 inhibited the signalling activity equally in all the culture media tested (see Figure 10).
Finally, the effects of WT-LRP5 and its mutants on Tph1 and 5-Htr1b expression were assessed using qPCR analyses. CHO cells were transfected with the LRP5 constructs and grown in either 10%FBS-DMEM, Wnt3a-CM or L1-CM. The gene expressions of Tph1 and 5-Htr1b were measured and presented as fold changes relative to the untreated control sample (pcDNA3.1+), normalised to β-actin. None of the LRP5 mutants affected Tph1 expression in the presence of 10%FBS-DMEM. When using Wnt3a-CM, only a change with the G1185R mutant was observed in comparison with L1-CM (p = 0.045) leading to a modest decrease of Tph1 expression relative to WT-LRP5 (p = 0.012). However, the statistical significance was lost after repetitions (p = 0.19).

LRP5 mutants did not affect 5-Htr1b expression in 10%FBS-DMEM. In the presence of Wnt3a-CM and compared to L1-CM, the 5'-Htr1b expression was increased in the C913fs (p = 0.034) and G1185R (p = 0.035) transfected cells, but did not differ from that in WT-LRP5 transfected cells. However, the 5'-Htr1b expression decreased with mutants L1149Q (p = 0.002) and R1036Q (p = 0.02) as compared to WT-LRP5, but the statistical significance of the latter was lost after repeats (p = 0.50).
6 Discussion

6.1 Haplotype association in femoral neck stress fractures (I)

Stress fractures present a serious overuse injury, which may have devastating consequences for skeletal health and, hence, an individual’s quality of life. They are common in athletes and military conscripts who are subjected to heavy exercise and strenuous mechanical loading. The genetic factors involved in the development of stress fractures are yet largely unexplored, and the aetiology is presumed to be multigenic. To date only three studies have been published on the subject (Chatzipapas et al. 2009, Välimäki et al. 2005, Yanovich et al. 2011).

Our study on the genetics of femoral neck stress fractures revealed an interaction between the CTR allele C and the VDR haplotype C-A, and the risk for stress fractures was nearly three times higher in conscripts who lacked either one or both of these as compared to carriers of both. Furthermore, the LRP5 haplotype A-G-G-C increased the risk for stress fractures by almost 3-fold, and the risk was nearly 4-fold in conscripts who had the LRP5 A-G-G-C haplotype in combination with the VDR haplotype C-A. These associations were mediated by low weight and BMI.

6.1.1 Previous research on the genetics of stress fractures

The three previous studies on the genetics of stress fractures focused on candidate genes ESR1, AR and VDR. Välimäki et al. (2005) analysed two polymorphic sites (XbaI and PvuII) on ESR1 and the CAG repeat on AR and found no associations for stress fractures. However, another study on AR reported that the risk for stress fractures was halved in military conscripts with less than 16 CAG repeats in exon 1, where the number of repeats varied from 6 to 31 (20.6 ± 4.3) in patients and from 11 to 32 in controls (20.0 ± 3.8) (Yanovich et al. 2011). The study on VDR examined four polymorphic restriction sites (FokI, BsmI, ApaI, TaqI) of which FokI and BsmI were found to be of interest. The f allele (absence of FokI site) increased the risk of stress fractures by 2.7-fold (95% CI 1.2–5.9, p = 0.014) and the B allele (presence of BsmI site) by 2.0-fold (95% CI 1.0–4.1, p = 0.053), and these alleles also associated with reduced T-scores (Chatzipapas et al. 2009). The association of the minor allele on FokI with stress fractures agrees with our results while the result on the BsmI site is not consistent with our findings.
However, the VDR results on bone parameters and/or fractures have been contradictory in general, and one of the possible contributors may be the small sample sizes as discussed below.

The sample sizes of all genetic studies on stress fractures have been small: the largest study to date was performed by Yanovich et al. (2011) with 171 stress fracture patients and 283 healthy controls. Välimäki et al. (2005) performed their studies on 15 conscripts with stress fractures and 164 controls and Chatzipapas et al. (2009) analysed 32 subjects and 32 controls. Finally, our study contained 72 conscripts with femoral neck stress fractures and 120 healthy controls. Because of the small sample sizes, the results of these studies should still be interpreted with caution and major conclusions have to wait until the results have been confirmed in replications in larger patient sets. There is evidence that genetic factors may affect BMD differently at specific sites (Salamone et al. 1996), hence it might be important to assess the genetics of stress fractures at one location at a time.

6.1.2 Genetic predisposition for fractures and/or reduced BMD

Though research on the genetics of stress fractures is just beginning, studies on the genetic susceptibility for osteoporotic fractures have already provided some clues on genetic determinants of bone. These studies have examined the associations between fractures or BMD and different bone-related candidate genes, including CTR, VDR and LRP5, which showed associations in our stress fracture study.

CTR mediates the inhibitory effect of calcitonin on bone resorption and is therefore important for maintaining bone homeostasis (Reginster 1993). Genetic studies on the association between the CTR polymorphism AluI and BMD have given conflicting results. The CC genotype has been reported to be more common in non-osteoporotic postmenopausal women than the TT genotype (Masi et al. 1998b, Nakamura et al. 1997). Furthermore the TT genotype has been associated with low BMD in the lumbar spine and femoral neck and shown to increase the risk for osteoporosis (Masi et al. 1998a, Masi et al. 2002, Tsai et al. 2003). In contrast, other studies have found an association between the CC genotype and reduced BMD both in women and men (Braga et al. 2000, Braga et al. 2002), and CC was also reported to be more frequent in men with hip or vertebral fractures than in control subjects (Braga et al. 2002). Our results are in line with those of the latter studies, suggesting a protective effect of the CTR allele C in combination with VDR C-A for femoral neck stress fractures. Our observation on
the interaction of CTR and VDR is supported by other studies. Low BMD and increased bone loss was detected in idiopathic arthritis patients with CTR TT and VDR ff genotypes (Masi et al. 2002), and polymorphisms in these genes have been associated with BMD in postmenopausal women (Bandres et al. 2005). However, as association studies do not identify causes or mechanisms, the nature of the interaction remains unknown. However, one of the possible explanations for the CTR and VDR interaction may be their role in maintenance of normocalcemia by inhibition of PTH production (Garfia et al. 2002). It is possible that this allele-haplotype affects PTH regulation and hence Ca levels.

VDR is also a receptor that affects bone biology. It binds 1,25(OH)_{2}D_{3} and is hence involved in the regulation of calcium and phosphate homeostasis, bone mineralisation and remodelling (Haussler et al. 1998). Similar to the CTR findings, the genetic association studies between VDR and BMD or fractures have produced controversial results (Eisman 1999, Li et al. 2010, Peacock 1995). Some of the positive associations have indicated that VDR genotypes or haplotypes associate with the risk of low BMD and fractures (Masi et al. 2002, Morrison et al. 1994, Uitterlinden et al. 2001), while other studies have shown the opposite (Houston et al. 1996, Salamone et al. 1996, Uitterlinden et al. 1996). To add to the controversy, a negative association between VDR polymorphisms and BMD have also been reported (Alahari et al. 1997, Spotila et al. 1996). Meta-analyses have become more common in the effort to resolve the association, but so far even these results have been disputable (Cooper & Umbach 1996, Fang et al. 2006, Gong et al. 1999, Ji et al. 2010, Uitterlinden et al. 2006). Small sample sizes, differences in the genetic background between patients and controls and/or environmental factors may account for the discrepancies in the results (Eisman 1999, Raisz 2005). Despite these controversies, the important role of the VDR remains a focus of bone biology.

Our results indicated that the association of the LRP5 haplotype and the LRP5-VDR interaction with femoral neck stress fractures were mediated via low weight and BMI, though the finding needs to be confirmed in further studies. The essential role of LRP5 in bone formation is well-recognised (Westendorf et al. 2004). Mutations within the gene affect bone disorders varying from osteoporosis to HBM (Gong et al. 2001, Hartikka et al. 2005, Little et al. 2002) while polymorphisms have been associated with BMD and BMC in the general population (Ferrari et al. 2004, Ferrari et al. 2005) and with osteoporosis, reduced BMD and fractures (Ferrari et al. 2005, van Meurs et al. 2006, van Meurs et al. 2008). Because mouse studies have indicated that Lrp5 mutations affect bone formation
sensitivity in response to normal mechanical loading (Akhter et al. 2004, Sawakami et al. 2006), it is possible that the $LRP5$ haplotype A-G-G-C alters bone remodelling in response to mechanical loading. In addition, it may be that the genetically set remodelling response of the low-weight conscripts is not efficient enough to adapt for the increased strain during military service, resulting in a higher risk for stress fractures in these conscripts. Many of the studies presented above identified genetic associations with reduced BMD. Association analyses between the candidate genes and BMD would have been interesting and possibly informative in our patient set as well, but unfortunately these values were not available for the study.

6.1.3 Body weight and bone density

Our findings are in line with previous studies suggesting that low weight (before and/or during military service) increases the risk of stress fractures (Armstrong et al. 2004, Brudvig et al. 1983). A close association between weight and bone density has been reported before (Krall & Dawson-Hughes 1993, Seeman et al. 1996). Body weight is considered as an important predictor of BMD (Lau et al. 2005) and high BMI has been found to associate with high BMD (Morin et al. 2009). Furthermore, $LRP5$ polymorphisms have also been associated with obesity (Guo et al. 2006), extending the role of $LRP5$ to weight regulation. Finally, our study is not the first to present weight as a mediator for an association between a genetic component and BMD. Body weight mediated the association between a $COL1A1$ genotype and bone density (Uitterlinden et al. 1998), and possibly also the association between a $COL1A1$ polymorphism and the age-related rate of bone-loss (Braga et al. 2000). More importantly, Vandevyver et al. (1997) detected an association between BMD and the $VDR$ genotype in non-obese women, and Dennison et al. (2001) observed an association between the $VDR$ genotype and BMD at the lumbar spine varied relative to birth weight.

Body weight was found to be the most important physical variable in our study, for reduced weight and BMI mediated the association of the $LRP5$ haplotype and the $LRP5$-$VDR$ interaction with stress fractures. Therefore, the discrepancy in the collection periods of the cases and the control groups could be considered as a limitation. However, the difference in weight and BMI was verified to be present also between the cases and their healthy contemporaries, indicating that the difference was not time-dependent. The verification was necessary, because the mean BMI and the number of overweight military conscripts has been constantly increasing during the past decades, while their
physical fitness has declined (Santtila et al. 2006). It would have been interesting to include and examine also other intrinsic factors (such as nutrition or exercise) that might have changed within the last 30 years, but unfortunately this data was not available for us.

6.1.4 Future directions

Our results suggest a genetic predisposition for femoral neck stress fractures, but the results should be interpreted with caution and replication of the study is needed to confirm the present findings. Future studies should be performed on larger sample sizes to gain more statistical power in the analyses, a suggestion that has also been made in other reports, and the genetic background of the cases and controls should be ensured. Furthermore, since association studies do not reveal the underlying cause or mechanism of stress fractures, functional studies are necessary to elucidate the relevance of the current genetic associations to femoral neck stress fractures. Our findings add to the novel research into the genetics of stress fractures, and suggest a role for CTR, LRP5 and VDR in the development of femoral neck stress fractures in individuals subjected to heavy exercise and mechanical loading. The present results can be applied to the design of future studies that will further unravel the genetics of stress fractures.

6.2 Mutations and variants associated with non-OI primary osteoporosis (II-III)

Non-OI primary osteoporosis is an early-onset bone disorder that manifests in reduced BMD and peripheral and/or vertebral compression fractures (Gordon et al. 2008, Ward LM 2003). Three mutations in LRP5 (A29T, C913fs, R1036Q) have been previously described in non-OI primary osteoporosis patients (Hartikka et al. 2005), and the two studies in this thesis (articles II and III) further support the role of LRP5 and Wnt signalling in the development of the disorder. In these studies two additional heterozygous missense mutations were detected in LRP5 (L1149Q and G1185R) and two variants observed in DKK1 (R120L) and WNT3A (K51R). The in vitro studies performed showed reduced Wnt signalling activity with two LRP5 mutants (C913fs, L1149Q) and the Wnt3a variant (K51R), suggesting a putative effect for these mutants. These effects were further supported by parallel results gained from our in vitro studies using Wnt1 (unpublished results).
In most of our families who were identified with mutations, the affected children’s phenotypes tended to be graver than those of their parents who also had the mutation. This tendency was observed especially in patients M13, OP4 and OP17, who had \textit{LRP5} (G1185R), \textit{WNT3A} and \textit{DKK1} variants, respectively. A similar trend for a more severe bone phenotype in offspring has been observed in families where heterozygous carriers of OPPG-causing \textit{LRP5} mutations present milder phenotypes i.e. primary osteoporosis and/or osteopenia as compared to OPPG (Gong \textit{et al.} 2001, Laine \textit{et al.} 2011). Reasons that might explain differences in the severity of the bone phenotypes and in the onset of osteoporosis in non-OI primary osteoporosis families include variations in mutation penetrance or the presence of some other predisposing genetic factors (Mejia-Gaviria \textit{et al.} 2010, Wang \textit{et al.} 2011). Furthermore, the disorder may be multigenic (Moron \textit{et al.} 2006) or multifactorial in nature.

\subsection*{6.2.1 Clinical considerations}

The non-OI primary osteoporosis patient sets have some limitations that need to be addressed. Firstly, the patient set I would have benefited from more thorough and uniform description of the clinical data. DNA samples were collected in the early 2000s from Germany and Canada, but unfortunately clinical information was not compiled concurrently. Collecting data afterwards was challenging, and consequently data was not available for three patients. Secondly, eighty Canadians with diagnoses of skeletal dysplasia, but no signs of osteoporosis, were used as controls for the patient set II in article III. These controls were available for the study at the time of sample collection and fulfilled the requirement of originating from the same geographical area as the patients. However, it would have been valuable to include also healthy individuals into the control group and in the genetic screening of the identified \textit{WNT3A} and \textit{DKK1} variants.

\subsection*{6.2.2 Location and significance of the variants}

The variants identified in our patients were located at coding regions of \textit{LRP5}, \textit{WNT3A} and \textit{DKK1}. Protein sequence alignments (Figure 6) showed that the variants affect conserved amino acid positions, which suggests their structural or functional importance. These were further examined by analysing the site of the variation using five computational programs (Adzhubei \textit{et al.} 2010, Bromberg \& Rost 2007, Capriotti \textit{et al.} 2005, Cheng \textit{et al.} 2006, Kumar \textit{et al.} 2009, Ng \\&
All LRP5 mutants, and the DKK1 variant, were found deleterious or possibly damaging, although with some differences in the prediction of severity. Only the WNT3A variant K51R was found to have no major impact on protein function. The discrepancies in the results for different variants may be explained by computational distinctions in approaches and/or emphasis between programs.

When considering LRP5 mutations, there is a tendency for different bone disease causing mutations to be located at certain propeller domains. All HBM associated mutations are located on the first propeller domain of LRP5, whereas the majority of the OPPG-related mutations are scattered on the second and third propellers, though they are also found on others (See Supplemental table 1). Furthermore, the non-OI primary osteoporosis causing mutations identified in our studies tended to locate at the fourth propeller domain. Yet, primary osteoporosis and osteopenia associated mutations have also been detected in other domains and on splice sites in heterozygous carriers of OPPG-causing LRP5 mutations (Gong et al. 2001, Laine et al. 2011). Because the LRP5 propeller domains are known to have distinct functions and to interact with specific components, the putative connection between the location of LRP5 mutations and the resulting disorders may also provide insight into the disease mechanism assuming that the mutation affects the interactive system.

The first and second LRP5/6 propeller domains are known to bind a certain class of Wnts (e.g. Wnt1 and Wnt9b) (Bourhis et al. 2010, Ettenberg et al. 2010, Tamai et al. 2000), and inhibitors such as Wise, SOST and Dkk1 (Bourhis et al. 2010, Itasaki et al. 2003, Semënov et al. 2005). The third and fourth propeller domains are shown to bind DKK1 (Mao et al. 2001, Zorn 2001) and another class of Wnts (e.g. Wnt3a in LRP6) (Bourhis et al. 2010, Ettenberg et al. 2010). However, there is evidence that different Wnts may bind LRP6 simultaneously on specific domains (Bourhis et al. 2010), and hence compete with DKK1 binding (Bourhis et al. 2010, Li et al. 2010). To conclude, LRP5 mutations potentially alter the interaction between LRP5 and other components depending on the site of mutation.

As for DKK1, the protein contains two cysteine-rich domains (Dkk_N and Dkk_C), that are conserved within the Dkk family (Krupnik et al. 1999). The C-terminal Dkk_C inhibits Wnt signalling by binding LRP5/6 (Brott & Sokol 2002, Li et al. 2002, Mao & Niehrs 2003, Semënov et al. 2001), whereas the N-terminal Dkk_N is not involved in this pathway (Korol et al. 2008). Instead it has been found to induce prechondral and axial mesoderm in synergy with BMP
antagonism and to function in trans to complement the activity of Dkk_C in embryonic functions (Korol et al. 2008). Furthermore, Dkk_N has been shown to have an independent signalling function and is likely to employ distinct and currently unknown receptors in some other pathway (Korol et al. 2008). The variant in our non-OI primary osteoporosis patient, R120L, is on Dkk_N, and, consistent with the above results, had no effect on Wnt signalling activity. Future studies are essential to reveal the functions of Dkk_N and the pathway in which it participates. Thereafter the R120L variant can be reexamined in light of this information.

Finally, because of the difficulties involved in the isolation and characterisation of the active form of the WNTs, their structures and specific functions are still unknown. However, Willert et al. (2003) were able to purify Wnt3a and to identify an essential palmitoylation site on the conserved Cys77. This modification is crucial for Wnt3a function and affects protein secretion and transport. The WNT3A variant K51R is located only 26 amino acids from this palmitoylation site and 106 amino acids from N-glycosylation sites, and in addition to these, Wnt3a may still contain even more modification sites that are currently unknown. (Willert et al. 2003)

6.2.3 The in vitro analyses

The possible effects of LRP5 mutants and WNT3A and DKK1 variants were examined in in vitro studies. Out of the four LRP5 mutations, two (C913fs and L1149Q) showed statistically significant reduction in Wnt signalling activity in the presence of Wnt3a-CM as compared to WT-LRP5. In addition, activity was decreased with mutant R1036Q. This mutation has been reported also in OPPG patients (Saarinen et al. 2010), which supports its significance in bone pathologies. Mutant G1185R did not seem to affect signalling activity, hence additional functional studies and potentially different experimental approaches are needed to identify the underlying mechanism. Our results are congruent with previous functional studies that OPPG-causing mutations reduce Wnt signalling activity (Ai et al. 2005a, Saarinen et al. 2010, Urano et al. 2009). Yet, other mechanisms have also been suggested, including impaired protein trafficking to the cell membrane (Ai et al. 2005a) or affected protein expression and/or interference with Mesd or the Wnt/Fzd complex (Crabbe et al. 2005). The HBM mutation G171V in our study also produced results consistent with previous research by showing activity that was close to that of WT-LRP5 (Balemans et al.
Finally, a follow-up study in which we used Wnt1 as an activator of the pathway, gave results highly similar to those gained using Wnt3a-CM (unpublished results). These results provide further support to the significance of our findings on reduced signalling activity as a consequence of non-OI primary osteoporosis causing mutations and their possible effect on bone formation and bone phenotype.

The Wnt3a variant (K51R-Wnt3a), like the LRP5 mutations, showed reduced signalling activity compared to the WT-Wnt3a, and thus may also contribute to the defective bone phenotype via impaired bone formation. Interestingly, Wnt3a was recently shown to activate also the non-canonical Wnt/JNK pathway and to promote osteoblast differentiation (Qiu et al. 2011), providing yet more possibilities to further study the effect of this mutant. No statistically significant differences in signalling activity were observed between the R120L-DKK1 and WT-DKK1, but the variant may exercise its influence through other signalling pathways and/or mechanisms that were not examined in the thesis (see the discussion above). Previous research supports the roles of DKK1 and WNT3A in bone pathologies such as MM (Boland et al. 2004, Qiang et al. 2008a, Qiang et al. 2008b, Qiang et al. 2008, Tian et al. 2003), but also in the development, differentiation and/or maturation of healthy osteoblasts (Boland et al. 2004, van der Horst et al. 2005). Thus, further research is required to resolve the remaining questions on the function of these mutants.

6.2.4 Bone, Lrp5 and serotonin pathway

A new perspective on Lrp5 and bone was brought to light by Yadav et al. (2008) who found that Lrp5 produced in the murine intestine could inhibit Tph1 expression, and consequently also 5-HT synthesis and bone formation. To address this new aspect we examined whether the LRP5 mutations causing non-OI primary osteoporosis could affect Tph1 and/or 5-Htr1b expression in an in vitro system. According to our results, only the L1149Q mutation reduced 5-Htr1b expression significantly in the presence of Wnt3a, but none of the LRP5 mutations showed a direct regulatory effect on Tph1 expression. Still, our in vitro results cannot be readily compared to the in vivo studies of Yadav et al. (2008), and the interplay between molecules that are expressed in two different tissues may be too intricate to be revealed by in vitro study alone. Thus, the 5-Htr1b finding, while interesting, remains tentative, and additional research with a different approach is required to decipher its biological importance.
Clinical studies have reported that 5-HT levels are affected in patients with HBM and OPPG, and the results have supported the effect of 5-HT on bone phenotype (Frost et al. 2010, Saarinen et al. 2010, Yadav et al. 2008, Yadav et al. 2010a). However, so far the sample sizes in all studies have been rather small and statistical significances and range of effects have varied (Frost et al. 2010, Saarinen et al. 2010, Yadav et al. 2008, Yadav & Ducy 2010, Yadav et al. 2010b). These discrepancies may be explained by different measurement techniques, but perhaps also by the effect of dietary tryptophan (as a source for Tph), which cannot be totally excluded from clinical studies (Mödder et al. 2010, Warden et al. 2010). In light of these results, it would have been interesting to assess the circulating 5-HT levels also from our non-OI primary osteoporosis patients, but unfortunately the blood samples were not available for us.

The specific mechanism by which 5-HT regulates bone formation is still unknown, and conflicting results have been published recently (Cui et al. 2011, Niziolek et al. 2011). A study by Cui et al. (2011) showed that osteocytic activation or inactivation of Lrp5 in mice produced high or low bone mass, respectively. No correlation was observed between bone mass and circulating 5-HT in ovariectomised mice, and treatment with a Tph1 inhibitor did not change bone markers or bone mass, although circulating 5-HT was accordingly decreased (Cui et al. 2011). However, another study detected no differences in circulating 5-HT levels in mice with HBM causing Lrp5 or Sost mutations (Niziolek et al. 2011). With the remaining questions and the emergence of conflicting results on serotonin signalling, the role of Wnt signalling in bone cannot be forgotten. In addition to the LRP5 studies (Ai et al. 2005a, Hartikka et al. 2005, Little et al. 2002, Urano et al. 2009), the significance of Wnt signalling is supported by genetic findings on other components of the pathway including mutations in SOST in HBM disorders (Piters et al. 2010, Semënov & He 2006) and the implication of DKK1 and Wnt3a in MM (Qiang et al. 2008a, Qiang et al. 2008b, Tian et al. 2003).

### 6.2.5 Future directions

Our studies on non-OI primary osteoporosis identified two disease causing mutations in LRP5 and two variants in WNT3A and DKK1, and further support the role of components of the canonical Wnt signalling pathway in the development of non-OI primary osteoporosis. When our current results are combined with those of Hartikka et al. (2005), altogether seven patients were
found with mutations or variants from an overall sample set of 40 patients. This would indicate that 17.5% of cases may be explained by variations in the components acting on the Wnt signalling pathway. The methodology of genetic research has developed rapidly during the past decade and the present methods of whole genome and exome sequencing would benefit also the study of non-OI primary osteoporosis genetics. Our *in vitro* studies suggested a possible effect of LRP5 mutants and WNT3A variant on Wnt signalling activity, but additional studies, especially on DKK1, are necessary to gain a better understanding of the influence and importance of these variants on bone biology. It should be noted that, the mutations may also affect other signalling pathways, such as serotonin signalling, and these too should be considered in future studies. Finally, as the disorder and findings are rare, the results cannot be readily applied in larger populations, and the significance of other underlying factors (i.e. other genetic and/or environmental factors) should not be overlooked as they may also contribute to the disease.
7 Conclusions and future perspectives

Our studies propose a genetic predisposition for femoral neck stress fractures and suggest that mutations in the components of the canonical Wnt signalling pathway contribute to the development of non-OI primary osteoporosis. In many ways, this is merely the beginning of identifying the genetics factors for both disorders, and more research is necessary to elucidate the underlying genetic complexity. The rapid development of methodology has broadened the possibilities in genetic studies: the emphasis is shifting towards larger population sizes and GWAS and meta-analyses, while candidate gene studies are replaced by more effective whole genome and exome sequencing. The challenges in future genetic research lie in processing and analysing the data that these new approaches provide. Expertise in bioinformatics will be instrumental in assessing and unravelling the true genetic findings among the large amount of data produced by the novel methods. Furthermore, compiling knowledge on functional genomics (including transcriptomics and proteomics) will also benefit and promote genetic research of bone disorders.

Research provides important information not only on specific disorders, but it also broadens the insight and knowledge on health and disease pathology in general. This thesis provides a basis for further research. Future studies hold the promise of better molecular level diagnostics of bone disorders such as stress fractures and non-OI primary osteoporosis. Another aim of the research is the development of new treatments. Research on bone and the canonical Wnt signalling pathway has already led to medicinal applications, which may eventually result in targeted molecular treatment. Lastly, studies on the interactions between bone, Lrp5 and the serotonin pathway have revealed a new horizon of bone biology, although many questions remain to be resolved before we can see the overall picture of bone and its determinants.
References


101


111


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Appendix
Supplemental table 1. LRP5 mutations and associated diseases.

<table>
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<th>Exon/Intron</th>
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<th>Predicted protein change</th>
<th>Mutation type</th>
<th>Location/propeller domain</th>
<th>Phenotype</th>
<th>Reference</th>
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<td>(Gong et al. 2001)</td>
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<td>(L12_L20)ins</td>
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<td>Phenotype</td>
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<td>(Ai et al. 2005)</td>
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<td>Osteopetrosis</td>
<td>(Wesenbeeck et al. 2003)</td>
</tr>
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<td>OPPG</td>
<td>(Ai et al. 2005)</td>
</tr>
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<td>(Qin et al. 2005)</td>
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<tr>
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<td>(Ai et al. 2005)</td>
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<td>(Nikopoulos et al. 2010)</td>
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<td>(Urano et al. 2009, van Meurs et al. 2006)</td>
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<td>(Toomes et al. 2004)</td>
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<td>(Ai et al. 2005)</td>
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<tr>
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<td>(Ai et al. 2005)</td>
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*retinopathy of prematurity, *autosomal dominant osteopetrosis type I, *NW_925106.1, *NCBI build 36.1, hg 18. Reference sequences used were NM_002335.2 and NP_002326.2 (GenBank).
References for the supplemental table 1


Original articles


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1141. Määttä, Tuomo (2011) Down syndrome, health and disability: A population-based case record and follow-up study

1142. Leskelä, Tarja (2011) Human δ opioid receptor Phc27 and Cys27 variants: The role of heteromerization and pharmacological chaperones in receptor processing and trafficking

1143. Karjalainen, Minna (2011) Genetic predisposition to spontaneous preterm birth: approaches to identify susceptibility genes

1144. Saaristo, Timo (2011) Assessment of risk and prevention of type 2 diabetes in primary health care

1145. Vuononvirta, Tiina (2011) Etäterveydenhuollon käyttöönotto terveydenhuollon verkostoissa


1155. Markkanen, Piia (2012) Human δ opioid receptor: The effect of Phc27Cys polymorphism, N-linked glycosylation and SERCA2b interaction on receptor processing and trafficking


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IN QUEST OF GENETIC SUSCEPTIBILITY TO DISORDERS MANIFESTING IN FRACTURES

ASSESSING THE SIGNIFICANCE OF GENETIC FACTORS IN FEMORAL NECK STRESS FRACTURES AND CHILDHOOD NON-OI PRIMARY OSTEOPOROSIS