Genetic variation in the LDL receptor-related protein 5 (*LRP5*) gene: Association with bone health and metabolic parameters

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ACADEMIC DISSERTATION

To be publicly discussed, with the permission of the Medical Faculty of the University of Helsinki, in the Folkhälsan lecture hall Arenan, Topeliuksenkatu 20, Helsinki, on February 18th 2011 at 12 noon.

Helsinki 2011

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ISBN 978-952-92-8543-3 (paperback)
ISBN 978-952-10-6803-4 (PDF)
http://ethesis.helsinki.fi

Unigrafia Oy
Helsinki 2011

Dedicated to all those PhD students
who struggle with their thesis and fear
that this day never (or eventually) comes…

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LIST OF ORIGINAL PUBLICATIONS

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I **Saarinen A**, Välimäki VV, Välimäki MJ, Löyttyniemi E, Auro K, Uusen P, Kuris M, Lehesjoki AE, Mäkitie O. The A1330V polymorphism of the low-density lipoprotein receptor-related protein 5 gene (LRP5) associates with low peak bone mass in young healthy men. *Bone* 2007;40:1006-1012.

II Lappalainen S, **Saarinen A**, Utriainen P, Voutilainen R, Jääskeläinen J, Mäkitie O. LRP5 in premature adrenarche and in metabolic characteristics of prepubertal children. *Clinical Endocrinology (Oxf)* 2009;70:725-731.

III **Saarinen A**, Saukkonen T, Kivelä T, Lahtinen U, Laine C, Somer M, Toiviainen-Salo S, Cole WG, Lehesjoki AE, Mäkitie O. LDL receptor-related protein 5 (LRP5) mutations and osteoporosis, impaired glucose metabolism and hypercholesterolaemia. *Clinical Endocrinology (Oxf)* 2010;72:481-488.

IV **Saarinen A**, Mäyränpää MK, Lehesjoki AE, Mäkitie O. Low-density lipoprotein receptor-related protein 5 (LRP5) variation in fracture prone children. *Bone* 2010;46:940-945.

Publication II also appears in the thesis of Saila (née Lappalainen) Laakso (2009).

ABBREVIATIONS

APC	adenomatous polyposis coli
ApoE	apolipoprotein E
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
BP	blood pressure
CK1a	casein kinase 1 alpha
CNV	copy number variation
COS-1	African green monkey kidney cells
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DKK	dickkopf
EGF	epidermal growth factor
FEVR	familial exudative vitreoretinopathy
GWA	genome-wide association
GSK3	glycogen synthase kinase 3β
HBM	high bone mass
HDL	high-density lipoprotein
HEK-293T	human embryonic kidney cells
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LEF1	lymphoid enhancer-binding factor 1
LRP5	low-density lipoprotein receptor-related protein 5
LRP6	low-density lipoprotein receptor-related protein 6

MLPA	multiplex ligation-dependent probe amplification
NMD	nonsense-mediated mRNA decay
OI	osteogenesis imperfecta
OPPG	osteoporosis pseudoglioma
PA	premature adrenarche
PBM	peak bone mass
PCR	polymerase chain reaction
SD	standard deviation
sFRP	secreted frizzled-related protein
SNP	single nucleotide polymorphism
SOST	sclerostin
TCF	T-cell specific transcription factor
UTR	untranslated region
VDR	vitamin D receptor
Wif-1	wnt inhibitory factor
Wnt	wingless-int
WT	wild type

ABSTRACT

Bone mass accrual and maintenance are regulated by a complex interplay between genetic and environmental factors. Recent studies have revealed an important role for the low-density lipoprotein receptor-related protein 5 (LRP5) in this process. The aim of this thesis study was to identify novel variants in the *LRP5* gene and to further elucidate the association of *LRP5* and its variants with various bone health related clinical characteristics.

The results of our studies show that loss-of-function mutations in *LRP5* cause severe osteoporosis not only in homozygous subjects but also in the carriers of these mutations, who have significantly reduced bone mineral density (BMD) and increased susceptibility to fractures. In addition, we demonstrated for the first time that a common polymorphic *LRP5* variant (p.A1330V) was associated with reduced peak bone mass, an important determinant of BMD and osteoporosis in later life. The results from these two studies are concordant with results seen in other studies on *LRP5* mutations and in association studies linking genetic variation in *LRP5* with BMD and osteoporosis.

Several rare *LRP5* variants were identified in children with recurrent fractures. Sequencing and multiplex ligation-dependent probe amplification (MLPA) analyses revealed no disease-causing mutations or whole-exon deletions. Our findings from clinical assessments and family-based genotype-phenotype studies suggested that the rare *LRP5* variants identified are not the definite cause of fractures in these children.

Clinical assessments of our study subjects with *LPR5* mutations revealed an unexpectedly high prevalence of impaired glucose tolerance and dyslipidaemia. Moreover, in subsequent studies we discovered that common polymorphic *LRP5* variants are associated with unfavorable metabolic characteristics. Changes in lipid profile were already apparent in pre-pubertal children. These results, together with the findings from other studies, suggest an important role for LRP5 also in glucose and lipid metabolism.

Our results underscore the important role of LRP5 not only in bone mass accrual and maintenance of skeletal health but also in glucose and lipid metabolism. The role of LRP5 in bone metabolism has long been studied, but further studies with larger study cohorts are still needed to evaluate the specific role of *LRP5* variants as metabolic risk factors.

INTRODUCTION

The first years of the 21st century were fascinating as new genetic data rapidly emerged after the release of the complete nucleotide sequence of the human genome (Lander et al. 2001, Venter et al. 2001, Levy et al. 2007). The research brought to light new mutations causing a variety of inherited disorders and genetic variants predisposing to common diseases. Similar developments took place in the research focused on finding the basic genetic determinants and mechanisms underlying normal bone metabolism and heritable bone disorders. The discovery and characterization of the low-density lipoprotein receptor-related protein 5 (*LRP5*) gene and its association with inherited bone disorders, the osteoporosis pseudoglioma (OPPG) syndrome and high bone mass (HBM) disorder (Gong et al. 2001, Little et al. 2002, Boyden et al. 2002), generated growing interest towards this gene and its role in normal bone mass development. The first association studies on *LRP5* polymorphic variants and low bone mineral density (BMD) (Ferrari et al. 2004, Koay et al. 2004, Mizuguchi et al. 2004, Urano et al. 2004) provided a promising platform for further studies.

This study was conducted to gain further knowledge about LRP5 and its role in bone metabolism. We decided to look for *LRP5* variants in Finnish patients with OPPG or skeletal fragility and to study the role of common polymorphic *LRP5* variants in skeletal characteristics. During the course of this thesis study, new data on the association between LRP5 and metabolic parameters emerged and therefore we expanded our studies to non-skeletal characteristics of OPPG and to patients with premature adrenarche (PA).

REVIEW OF THE LITERATURE

1. Bone

A newborn child has 270 bones, which grow and merge and eventually form the 206 bones that are in the adult human body. They act as a support for the rest of the body and enable movement together with muscles, tendons and joints. Bones also provide protection for vital organs such as the brain, heart and lungs. On the microscopic level bones have numerous vital metabolic functions, such as production of blood cells (haematopoiesis) and storage of minerals and fatty acids (Clarke 2008). Bone also acts as an endocrine organ by secreting osteocalcin, a hormone that regulates glucose metabolism and insulin sensitivity, and FGF23, a hormone involved in phosphate homeostasis (Shimada et al. 2004, Ferron et al. 2010).

1.1. Bone structure

The adult human skeleton is composed of cortical bone and trabecular bone. Different bones and skeletal sites within bones have different ratios of cortical to trabecular bone. Cortical bone is dense and solid and surrounds the marrow space. It accounts for most of the total bone mass of an adult skeleton. Bone marrow and most of the blood vessels are located in the trabecular bone, found at the ends of long bones and inside individual vertebrae. Unlike the dense cortical bone, the trabecular bone is composed of a honeycomb-like network of rod-like and plate-like elements. Among its many functions, trabecular bone is the place where haematopoiesis occurs. (Clarke 2008)

1.2. Bone cells

Bone is a constantly changing living tissue where new bone is formed and old bone is broken down throughout the human life. New bone is formed by osteoblasts, single-nucleated cells that are differentiated from multipotential mesenchymal stem cells. These stem cells also give rise to adipocytes (fat cells), myoblasts (muscle cells), chondrocytes (cartilage forming cells), and bone marrow stromal cells (Minguell et al. 2001). Osteoclasts, which are the bone degrading cells, are in turn large multi-nuclear cells that originate from differentiated and fused monocyte stem cells, the same progenitor cells that give rise to macrophages (Boyle et al. 2003). Osteoblasts and osteoclasts are constantly degrading old and making new bone in a process called bone remodeling (Figure 1). In childhood, when bones grow in length and width, the anabolic function of osteoblasts is greater than the catabolic function of osteoclasts.

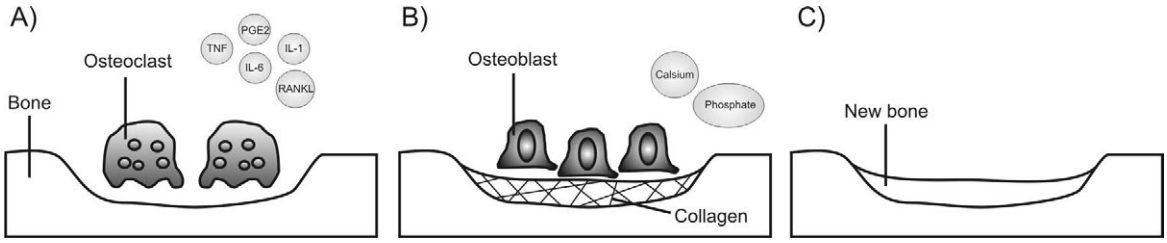

Figure 1. *Bone remodeling (a) Cytokines and prostaglandins activate osteoclasts to remove minerals and collagen from the bone surface. (b) They form a cavity, which is replaced by a new collagen network synthesized by osteoblasts. (c) Mineralization takes place when calcium and phosphate surround the collagen network.*

The most abundant (>90-95%) cell type in bone is the osteocyte, a single-nucleated cell that differentiates from osteoblasts (Bonewald and Johnson 2008). Osteocytes are dispersed throughout the mineralized matrix and connected to each other and other cells through dendrites. The complex function of the osteocytes is not yet fully understood, but it has long been thought that they might work as mechano-sensory receptors and function actively in bone turnover. Several studies have shown that a protein called sclerostin is expressed solely in the osteocytes (Balemans et al. 2001, Winkler et al. 2003, van Bezooijen et al. 2004). The essential role of osteocytes in the regulation of bone formation was further highlighted in recent studies showing that sclerostin works as an inhibitor of the Wnt signaling pathway (Li et al. 2005, Semenov et al. 2005) and is therefore a potential target for drug treatment against osteoporosis (Li et al. 2009, Eddleston et al. 2009).

1.3. Bone density

The fragility risk and the overall well-being of bones usually correlate with bone mineral density (BMD) and bone mineral content (BMC). BMD (g/cm^2) and BMC (g) are clinical values which can be measured using dual energy X-ray absorptiometry (DXA). BMD values are given as T-score or Z-score values, indicating the number of standard deviations (SD) the measured BMD differs from the mean reference value. The T-score value compares the measured BMD value with a BMD value of a 20 to 40 year old ethnic and gender matched healthy control population, whereas the Z-score indicates in SD units how much the measured BMD value differs from the average BMD of an age, sex, and ethnicity matched control population. According to The World Health Organization (WHO) definition, a BMD T-score -1.0 SD or higher is considered normal (Kanis et al. 1994). In post-menopausal women and in men over 50 years old, these groups having the highest risk of osteoporosis, the T-score values are used to predict fracture risk. The Z-scores are used when measuring BMD in children, pre-menopausal women or in men less than 50 years old as in such cases T-scores are not reliable (Carey and Delaney 2010).

The amount of bone mass varies during different phases of human life. Skeletal development begins at early stages of fetal development and bone mass accrual continues until peak bone mass (PBM) is attained in late adolescence. The bones reach their final length at the age of 16 to 18 years, but BMD still increases after that and PBM is reached only some years later. After that bone mass remains fairly stable for several years until it starts to decrease gradually (Peacock et al. 2002). After menopause bone loss becomes significantly more rapid, due to reducing estrogen levels and loss of the protective effect of this hormone against bone loss (Kiel et al. 2008). PBM is highly regulated by hereditary factors. It is estimated that as much as 80% of bone mass is determined by genes and only 20% can be explained by environmental factors (Howard et al. 1998, Peacock et al. 2005). Healthy life style factors, such as exercise, nutrition and healthy diet help in building and maintaining good bone health. The interaction between these lifestyle factors and genes that participate in bone metabolism and fracture risk determine the inter-individual variability of BMD.

1.4. Vitamin D

The human body contains approximately 1 kg of calcium, 99% of which is stored in the bones. Therefore a sufficient amount of calcium in needed in our diet. However, calcium cannot be absorbed from the gut without the help of vitamin D, a vitamin vital for maintaining good bone health. Vitamin D precursor 7-dehydrocholesterol is produced in the inner layers of the skin with the help of ultraviolet light from the sun. The 7-dehydrocholesterol is processed into pre-vitamin D cholecalciferol (D_3), which is circulated in the bloodstream to the liver and finally to the kidneys where it is processed into calcitriol, a physiologically active form of vitamin D (Lehmann and Meurer 2010).

In cells calcitriol binds to the nuclear vitamin D receptor (VDR) and activates its function as a transcription factor (Baker et al. 1988). VDR is expressed in most tissues and it has numerous vital functions throughout the human body. In addition to regulating the intestinal calcium absorption in skeletal metabolism, it also plays an important role in other metabolic pathways such as the immune system, and cell proliferation and differentiation (Uitterlinden et al. 2004a).

The amount of vitamin D processed in the skin may not alone be sufficient to maintain adequate calcitriol levels in the bloodstream. This is especially the case in the Nordic countries as the amount of sunlight is minimal during the winter months. In particular, people wearing clothing that covers the whole body and people with dark complexions are at a very high risk of vitamin D deficiency. Nutritional sources of vitamin D are

scarce and include fish and fortified dairy products. Vitamin D supplementation is recommended to prevent vitamin D insufficiency.

2. Bone disorders

An unbalanced diet can lead to severe health problems and can also affect bone health. Severe vitamin D and calcium deficiency can lead to rickets, a disease characterized by insufficient endochondral bone mineralization, impaired bone growth and deformities. Rickets is a common disease in developing countries especially in children, but it is also seen in adults and known as osteomalacia. Insufficient vitamin D intake also contributes to sub-optimal bone mass development and risk of fractures (Lips et al. 2008). Other environmental factors, such as lack of physical exercise and smoking, can also affect bone health (Ducher et al. 2008, Kanis 2008).

Genetic factors have an important role in the development of a large number of bone disorders. The etiology of some common bone diseases is multifactorial: environmental and lifestyle factors together with multiple genes define an individual's risk of getting the disease. On the other hand, some rare skeletal disorders are inherited as Mendelian traits where a specific mutation or mutations in a single gene cause the disease.

2.1. Osteoporosis

The most common bone disorder worldwide is osteoporosis. It affects millions of people every year and creates a major burden to the health-care system. In Finland, approximately 400 000 people suffer from osteoporosis and it causes 30 000 to 40 000 osteoporotic fractures yearly. As in many developed countries, the numbers have been continuously rising as a consequence of increased longevity (Kiel et al. 2008). In addition, lifestyle, nutrition and exercise habits have changed tremendously, especially in children, and it has been estimated that this can elevate the number of osteoporosis cases in the future (Harvey et al. 2008). Postmenopausal osteoporosis, the most common form of primary osteoporosis, is a multifactorial disorder as it is caused by a combination of genetic and environmental factors (Kiel et al. 2008). Secondary osteoporosis, on the other hand, is caused as a secondary effect due to various medical or pharmacological factors (Hamdy 2008). For example, patients with nutritional or gastrointestinal disorders (e.g. Crohn's disease and celiac disease) or patients taking anti-cancer drugs or glucocorticoids have an increased risk of osteoporosis.

WHO defines osteoporosis as BMD or BMC less than 2.5 SD below the young adult mean in the population (Kanis 1994). Osteoporotic bones have low bone mass and microarchitectural deterioration, which leads to enhanced bone fragility and increased

fracture risk. The majority of osteoporotic fractures occur in the wrist, hip and spine. (Harvey et al. 2008)

In recent years, a large number of genes have been associated with osteoporosis. At least 15 genes can be regarded as confirmed osteoporosis genes and even more are considered as promising candidate genes. The number of these genes is still rising as new susceptibility loci are found. Interestingly, the main genes and proteins are clustered in the same biological pathways, one of them being the focus of this thesis, the Wnt/β-catenin pathway. Table 1 summarizes some of the most promising pathways and candidate genes.

2.2. Osteogenesis Imperfecta (OI)

Osteogenesis Imperfecta (OI) is one of the most extensively studied genetic bone disorders. The name refers to the pathological mechanism of the disease, incomplete formation of bone. To date, at least eight clinically different OI subtypes have been described and the symptoms vary even between individuals with a similar OI subtype. The clinical feature common to all OI patients is the fragility of bones, which usually leads to recurrent fractures, severe deformities and sometimes even death at early stages of life. Other clinical features include loose joints, short stature, respiratory and hearing problems, blue sclera, and dentinogenesis imperfecta (aberrant tooth development). It is estimated that approximately 6-7 in 100 000 people are affected by OI or one of its subtypes each year. In Finland there are approximately 400 patients with OI.

Most OI patients have dominant mutations in the genes coding for type I collagen (*COL1A1, COL1A2*), a protein essential for strengthening and supporting various tissues, including bone, cartilage and skin (Rauch and Glorieux 2004). In some rare cases, a recessive form of OI has been reported as a consequence of mutations in the *CRTAP*, *LEPRE1*, *PPIB*, *SERPINH1,* and *FKBP10* genes encoding CRTAP, P3H1, CyPB, HSP47, and FKBP65 collagen-modifying enzymes or chaperon proteins (Morello et al. 2006, Cabral et al. 2007, van Dijk et al. 2009, Alanay et al. 2010, Christiansen et al. 2010).

2.3. Osteoporosis pseudoglioma (OPPG) syndrome

Osteoporosis pseudoglioma syndrome (OPPG) is a genetic disorder characterized by severe juvenile onset osteoporosis and congenital or early-onset visual loss. The bone phenotype is caused by disruption of bone mass accrual and reduced osteoblast proliferation during growth whereas the visual loss is caused by extensive eye vascularization due to disrupted macrophage function during early eye development (Gong et al. 2001, Kato et al. 2002). While the disease was characterized in the 1960's

the causative genetic defect was not discovered until 2001 when Gong and co-workers showed that mutations in the *LRP5* gene cause OPPG (Gong et al. 2001).

Table 1. *Summary of some of the most promising osteoporosis-associated genes.*

Gene	Protein	Status	Method	Ref.
COL1A1	Type 1 collagen	Confirmed	CGAS+MA	1,2
ESR1[*]	Estrogen receptor 1	Confirmed	CGAS+GWAS+MA	3-5
ESR2[*]	Estrogen receptor 2	Confirmed	CGAS	4,6,7
GRP177[**]	G-protein-coupled receptor 177	Confirmed	MA	5
ITGA1	Integrin alpha 1	Confirmed	CGAS+MA	8,9
LRP4[**]	Low-density lipoprotein receptor-related protein 4	Confirmed	GWAS+MA	3,5
LRP5[**]	Low-density lipoprotein receptor-related protein 5	Confirmed	CGAS+GWAS+MA	5,10,11
SOST[**]	Sclerostin	Confirmed	CGAS+GWAS	12,13
SOX6	SOX transcription factor 6	Confirmed	GWAS+MA	5,14
SP7	Zink finger protein osterix	Confirmed	GWAS+MA	5,13
SPP1	Secreted phosphoprotein 1	Confirmed	CGAS+MA	9,15
TNFRSF11[***]	Receptor activator of NF-kappa-B ligand (RANKL)	Confirmed	CGAS+GWAS+MA	3,5
TNFRSF11A[***]	Receptor activator of NF-kappa-B (RANK)	Confirmed	CGAS+GWAS+MA	5,13
TNFRSF11B[***]	Osteoprogerin (OPG)	Confirmed	CGAS+GWAS+MA	3,5,10
VDR[****]	Vitamin D receptor	Confirmed	CGAS+GWAS+MA	3,16,17
CTNNB1[**]	Beta-catenin	Suggestive	MA	5
FOXC2[**]	Forkhead box C2	Suggestive	CGAS+GWAS	5,18
MARK3	Microtubule affinity-regulating kinase 3	Suggestive	GWAS+MA	13
SFRP4[**]	Secreted frizzled-related protein 4	Suggestive	GWAS	19
SPTBN1	Spectrin beta non-erythrocytic 1	Suggestive	MA	5
TGFBR3	Transforming growth factor beta receptor 3	Suggestive	GWAS	20
UGT2B17[*]	Uridine diphosphoglucuronosyltransferase	Suggestive	GWAS	21
ZBTB40	Zink finger and BTB domain containing 40	Suggestive	GWAS+MA	3,5

*CGAS, candidate gene association study; GWAS, genome-wide association study; MA, meta-analysis. The corresponding pathways are marked with an asterisk: *) the estrogen endocrine pathway, **) the Wnt/beta-catenin signaling pathway, ***) the RANKL/RANK/OPG pathway, ****) the vitamin D endocrine pathway. Referred publications: 1) Steward et al. 2006, 2) Husted et al. 2009, 3) Styrkarsdottir et al. 2008, 4) Wang et al. 2008, 5) Rivadeneira et al. 2009, 6) Rivadeneira et al. 2006, 7) Massart et al. 2009, 8) Lee et al. 2007, 9) Richards et al. 2009, 10) Richards et al. 2008, 11) van Meurs et al. 2008, 12) Uitterlinden et al. 2004b, 13) Styrkarsdottir et al 2009, 14) Liu et al. 2009, 15) Willing et al. 2003, 16) Fang et al. 2005, 17) Moffett et al. 2007, 18) Yamada et al. 2006, 19) Cho et al. 2009, 20) Xiong et al. 2009, 21) Yang et al. 2008.*

OPPG is inherited as an autosomal recessive trait and is an extremely rare disorder, the estimated incidence being as low as 1 in 2 000 000 and the carrier frequency of 1 per 700 (Ai et al. 2005a). Estimates from these numbers suggest that as many as 7 500 people in Finland could be *LRP5* mutation carriers. Identification of these individuals is important since findings in several studies suggest a reduced BMD in OPPG mutation carriers (Gong et al. 2001, Lev et al. 2003).

2.4. High bone mass disorder (HBM)

The autosomal dominant high bone mass (HBM) disorder was originally discovered through a surprising coincidence where a teenage girl from Nebraska USA survived a car collision without breaking a single bone. Her bones were subsequently shown to be about 50% more dense than normal and therefore resistant to fractures. This started an intensive study among the family as several family members were found to have similarly increased BMD. The causative gene defect was found by genetic linkage studies (Johnson et al. 1997, Little et al. 2002, Boyden et al. 2002). Surprisingly the HBM gene turned out to be *LRP5*, the same gene that was one year earlier associated with the low bone mass phenotype OPPG.

2.5. Familial exudative vitreoretinopathy (FEVR) type 4

Familial exudative vitreoretinopathies (FEVRs) are a diverse group of disorders characterized by incomplete vascularization of the retina (Benson 1995). Mutations in *NDP*, *FZD4*, *LRP5* and *TSPAN12* genes, coding for proteins in the Norrin/β-catenin pathway, have been reported to cause different forms of FEVR (Poulter et al. 2010). Autosomal dominant and autosomal recessive forms of FEVR type 4 are caused by mutations in *LRP5* (Toomes et al. 2004). In addition to ocular changes, patients with FEVR type 4 have reduced BMD. This has led to speculation suggesting that FEVR type 4 and OPPG are part of a single phenotypic spectrum with both ocular and bone manifestations (Qin et al. 2005).

2.6. Treatment possibilities for osteoporosis and other bone disorders

As there is no effective treatment for most genetic bone disorders the patients are treated with the same drugs as osteoporosis patients. In addition to exercise and nutritional recommendations, there are several pharmacological agents affecting bone metabolism. Antiresorption drugs, such as bisphosphonates, inhibit bone resorption by osteoclasts whereas some anabolic agents, such as teriparatide, stimulate osteoblast activity, thus enhancing bone formation (Kleerekoper 2008).

Recent studies on inhibitory proteins of the Wnt/β-catenin signaling pathway have revealed potential targets for treatment of osteoporosis and other bone related disorders (Roux 2010). A more detailed summary of these future scenarios will be discussed in the next section.

3. The Wnt signaling pathway

The Wnt signaling pathway is one of the most extensively studied pathways in biology. It is the focus of a broad spectrum of research in embryogenesis, cancer research, stem cell studies, and bone metabolism. The Wnt signaling pathway is highly conserved across species and it regulates various essential cellular functions, for example embryonic development, homeostasis, and cell differentiation (Angers and Moon 2009). Although the function and relevance in many of these areas is still largely unclear, its crucial role in bone metabolism is undeniable.

The Wnt signaling pathway consists of at least four different pathways, the canonical pathway, the planar cell polarity (PCP) pathway, the Wnt/Ca^{2+} pathway, and the protein kinase A pathway (Semenov et al. 2007). The canonical Wnt signaling pathway, being the most studied one, is activated through β-catenin, whereas the non-canonical pathways are β-catenin independent. Problems in the Wnt signaling pathway, caused by mutations or other environmental factors, can lead to homeostatic disequilibrium, severe developmental abnormalities or cancer (Moon et al. 2004).

3.1. Wnt proteins

Wnt proteins are secreted extracellular glycoproteins that act as ligands in the Wnt signaling pathway. The name Wnt comes from a combination of *Drosophila* wingless (wg) and a murine int-1 proto-oncogene. To date, nineteen Wnt genes have been identified in the human genome. Of these, Wnt1, Wnt3A, and Wnt8 are, in the current understanding, the ligands that activate the canonical Wnt signaling pathway (Van Amerongen et al. 2008). From here onward, I will focus mainly on the canonical Wnt/β-catenin signaling pathway.

3.2. Genes in the canonical Wnt/ beta-catenin signaling pathway

In the presence of Wnt ligands, the canonical Wnt/β-catenin signaling cascade is activated through dephosphorylation of intracellular β-catenin. The Wnt ligands bind to the LRP5/6 and Frizzled complex located in the cell membrane. This induces a breakdown of the GSK-3β inhibitory complex, which in turn stabilizes β-catenin and allows its accumulation in the cytoplasm and nucleus. In the nucleus, β-catenin activates transcription of the desired target genes. A schematic diagram of the Wnt/β-

catenin signaling pathway is presented in Figure 2. The main genes and protein complexes are described in detail below.

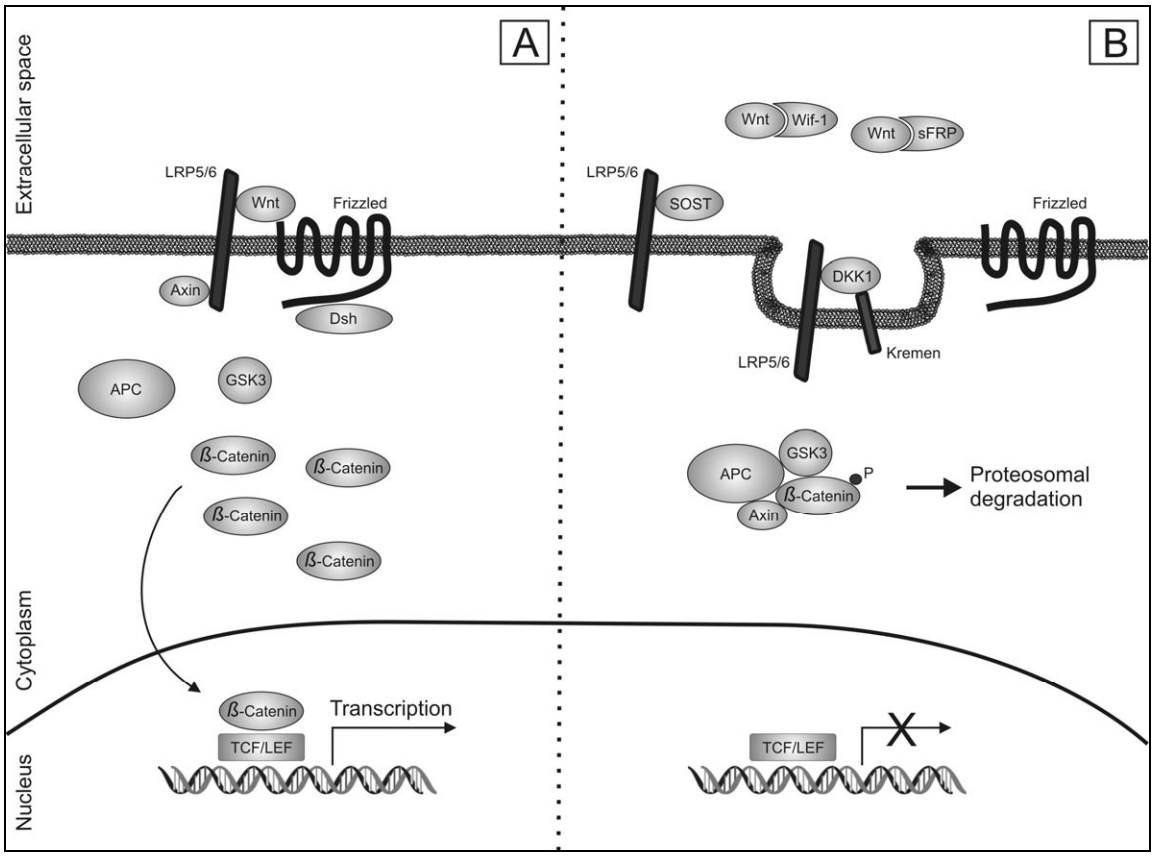

Figure 2. *A schematic drawing of the Wnt/beta-catenin signaling pathway.*
A) Activation of the pathway is initiated when Wnt associates with Frizzled and LRP5/6. This causes activation of intracellular Dishevelled (Dsh) which, in turn, inhibits GSK3. ß-catenin is no longer phosphorylated and is thus stabilised and translocated to the nucleus where it induces transcription via the TCF/LEF family of transcription factors.
B) Inhibition of the pathway is initiated by LRP5/6 binding inhibitory proteins (DKK1 and SOST) or Wnt-binding proteins (sFRPs and Wif-1). For example, DKK1 interaction with LRP5/6 and Kremen triggers endocytosis which prevents the formation of LRP5/6–Wnt–Frizzled complex. Axin brings together the proteins that promote ß-catenin phosphorylation, enabling ß-catenin degradation and inhibition of the canonical pathway.

3.2.1. LRP5/6 and Frizzled complex

Frizzled proteins were the first identified receptors shown to interact with the Wnt ligands (Bhanot et al. 1996). Structurally, Frizzled is a membrane protein with seven transmembrane loops and a cytoplasmic tail. In the extracellular amino-terminus, Frizzled has a cysteine-rich binding site for Wnts. Wnt ligands, together with Frizzled, bind to a specific domain of LRP5/6, activating signal transduction (Figure 2A).

LDL receptor-related proteins 5 and 6 (LRP5 and LRP6) are single-pass transmembrane proteins with 71% homology. The extracellular part of these proteins is comprised of four repeating epidermal growth factor (EGF) repeats and YWTD propeller domains, which serve as a binding site for the Wnts. In the intracellular C-terminus, they have several PPPS-motifs, which are phosphorylated by GSK-3β after Wnt activation. In addition, cytoplasmic proteins Dishevelled and Axin are required for this phosphorylation, which in turn deactivates the GSK-3β complex and stabilizes β-catenin (Figure 2A).

3.2.2. GSK-3beta complex

The key components of the GSK-3β complex are the following proteins: glycogen synthase kinase GSK-3β (GSK3), adenomatous polyposis coli protein (APC), casein kinase 1 alpha (CK1a), and Axin. Of these, GSK3 and CK1a have a phosphorylating kinase activity, which is active in the absence of Wnt proteins. During this period, the cytoplasmic β-catenin is phosphorylated and transported to the proteasome complex for degradation (Figure 2B). This degradation continues constantly until Wnt proteins activate the signaling cascade and demolish the GSK-3β complex.

The genes coding for these GSK-3β complex proteins have been widely studied because of their role in the development of some cancer types. For example, mutations in the *APC* gene, also known for its role as a tumor suppressor gene, can cause colorectal cancer (Morin et al. 1997). More recently, an interesting link was proposed between increased GSK3 activity and common diseases such as Alzheimer's disease and diabetes (De Ferrari & Inestrosa 2000, Kaidanovich & Eldar-Finkelman 2002).

3.2.3. Beta-catenin and TCF/LEF1 complex

β-catenin (armadillo in *Drosophila*) is encoded by the *CTNNB1* gene and it has a central role in the canonical Wnt signaling pathway. The breakdown of the GSK-3β complex in the presence of Wnt proteins dephosphorylates and activates the cytoplasmic β-catenin. It then accumulates to the nucleus and binds to specific transcription factors initiating the transcription of Wnt target genes (Figure 2A).

The lymphoid enhancer-binding factor 1 (LEF1) and T-cell specific transcription factors (TCF) form a family of DNA binding transcription factors that, together with β-catenin, activate a number of Wnt target genes. Many of these genes have been identified through experimental approaches and most of them have a role in development or tumorigenesis.

As with the GSK-3β complex, defects in the β-catenin signaling or TCF/LEF1 complex can lead to severe outcomes. The *CTNNB1* gene can function as an oncogene, as mutations in *CTNNB1* can increase β-catenin production and cause cancer (Morin et al. 1997, Moon et al. 2004). Interestingly, common polymorphisms in one of the TCF family genes, *TCF7L2*, were associated with type 2 diabetes (Grant et al. 2006).

3.3. Inhibition of the canonical Wnt/ beta-catenin signaling

Excess activation of the canonical Wnt signaling is prevented by various extracellular inhibitory proteins. Dickkopfs (DKK) and sclerostin (SOST) bind to the Wnt binding domain of LRP5/6, preventing Wnts from activating the signaling (Figure 2B). On the other hand, secreted frizzled related proteins (sFRPs) and Wnt inhibitory factor (Wif-1) bind directly to Wnt ligands and prevent their binding to the receptor (Figure 2B). The presence of these inhibitory proteins and the knowledge about Wnt signaling in various disorders has introduced a promising platform for potential treatment modalities against these illnesses. The main inhibitory proteins and their role as possible therapeutic agents in various diseases are discussed next in detail.

3.3.1. Dickkopf (DKK1)

In the human genome, the Dickkopf (DKK) family consists of four members, *DKK1*, *DKK2*, *DKK3*, and *DKK4*. Of these, DKK1 is probably the most extensively studied and it functions as a negative regulator of the canonical Wnt signaling. DKK1 is expressed in various neural and mesenchymal tissues during embryonic development and it has a central role in head development (Glinka et al. 1998). In adult tissues it is expressed in osteoblasts and osteocytes and therefore has an important role also in bone metabolism (Zhang et al. 2004). Recent studies have shown that over-expression of DKK1 in transgenic mice results in decreased bone mass through reduced numbers of osteoblasts (Li et al. 2006). On the other hand, heterozygous *Dkk1*$^{+/-}$ deficient mice have increased bone formation and bone mass (Morvan et al. 2006).

DKK1 is a soluble protein that binds to the LRP5/6 receptor together with a protein called Kremen (Mao et al. 2002). This protein complex is removed from the plasma membrane by endocytosis, preventing Wnt ligand binding (Figure 2B). It is speculated that this process disrupts the dephosphorylation of β-catenin through Axin malfunction and that way inhibits the Wnt signaling (Kawano and Kypta 2003).

Recent studies have reported DKK1 as a potential target in the development of drugs against myeloma (Yaccoby et al. 2007). A DKK1 binding antibody was shown to decrease bone resorption and increase BMD in mice with induced myeloma, suggesting a significant anabolic effect on bone.

3.3.2. Secreted Frizzled related proteins (sFRPs)

Secreted Frizzled related proteins (sFRPs) are a diverse group of proteins that block the Wnt signaling by binding to Wnt proteins and preventing them from interacting with Frizzled (Figure 2B). As in Frizzled, the sFRPs have a cysteine rich domain which is likely to serve as the binding site for Wnts. This domain might also interact with itself and form a non-functional complex with Frizzled (Bafico et al. 1999).

Possible therapeutic approaches against altered bone metabolism might emerge from studies with sFRPs, as some of them contribute to the development of skeleton and bone formation. In addition, the Wnt binding approach has shown to be promising as antibodies against Wnt-1 and Wnt-2 induced apoptosis in human cancer cells (He et al. 2004, You et al. 2004).

3.3.3. Sclerostin

Sclerostin is a soluble protein which regulates bone mass by inhibiting Wnt signaling. It binds to the extracellular domain of LRP5/6 and disrupts Frizzled/LRP complex formation (Figure 2B) (Semenov et al. 2005). Sclerostin is encoded by the *SOST* gene, which is mutated in sclerosteosis and Van Buchem disease (Balemans et al. 2001 and 2002). Sclerostosis is an autosomal recessive disorder caused by a loss-of-function mutation in *SOST* and is characterized by dramatically increased bone density (Balemans et al. 2001). Van Buchem disease resembles sclerostosis, but is clinically less severe. It is caused by a large homozygous deletion in the regulatory area required for transcription of *SOST* (Balemans et al. 2002). *SOST* is expressed in osteoblasts and osteocytes and mice over-expressing *SOST* show severe bone loss, indicating an important role for *SOST* in bone homeostasis (Winkler et al. 2003).

The high bone mass phenotype in patients with sclerosteosis and Van Buchem disease, together with experiments in mice, strongly support the idea that interfering with SOST/LRP binding might be a potential strategy when developing treatment for osteoporosis and other low bone mass phenotypes. In fact, studies on rodents and monkeys treated with an anti-sclerostin antibody resulted in increased bone formation, bone mass and bone strength (Li et al. 2009, Eddleston et al. 2009, Ominsky et al. 2010).

4. Low-density lipoprotein receptor-related protein 5 (LRP5)

4.1. LRP5 genomic structure and homology

Human *LRP5* spans approximately 136 kb on the long arm of chromosome 11 at position 11q13.4. The gene consists of 23 exons with flanking 5' and 3' UTR regions and the total length of the transcript is 5124 basepairs. The translation initiation codon ATG is located in the first exon.

The LRP5 protein is fairly homologous among species. LRP5 has an amino acid similarity of 99.7% with chimpanzee (*Pan troglodytes*), 95.0% with rat (*Rattus norvegicus*), 94.3% with mouse (*Mus musculus)*, 76.4% with zebrafish (*Danio rerio*), and 46.1% with fruit fly (*Drosophila melanogaster*). Interestingly, no similarity to human *LRP5* is found in dogs (*Canis familiaris*), pigs (*Sus scrofa*), or worms (*Caenorhabditis elegans*).

4.2. LRP5 protein structure and expression

LRP5 consists of 1615 amino acids. After protein synthesis, LRP5 is folded in the ER and transported to the cell membrane with the help of chaperone protein MESD (Culi et al. 2003, Hsieh et al. 2003, Culi et al. 2004). LRP5 is embedded in the membrane with most of its structure in the extracellular matrix (Figure 3). This extracellular part is responsible for Wnt/Frizzled complex binding, whereas the cytoplasmic tail is involved in GSK-3β complex inactivation. Figure 3 illustrates the different LRP5 domains and their functions.

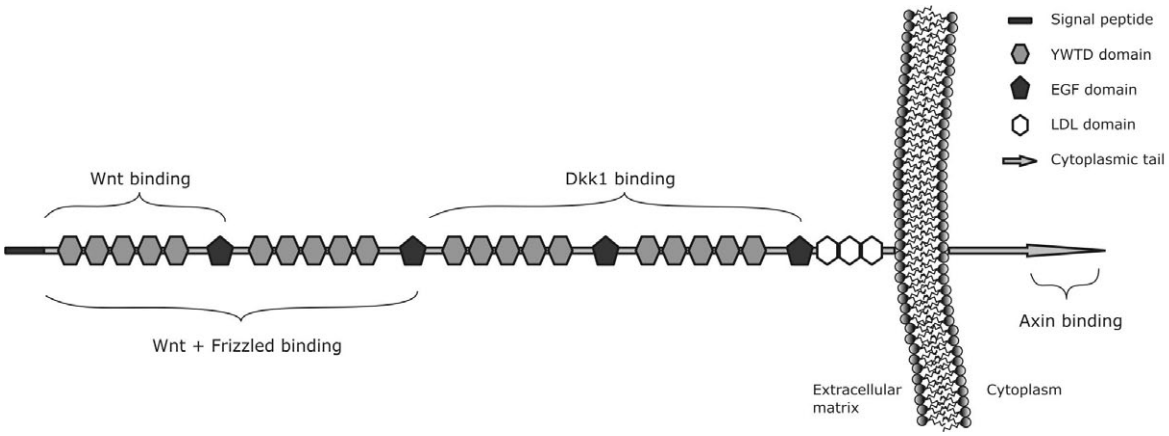

Figure 3. *A schematic diagram of LRP5 and its functional domains. An N-terminal signal peptide is followed by four alternating epidermal growth factor (EGF) and YWTD propeller domains and three cysteine-rich LDL domains. The transmembrane domain is followed by a short intra-cellular domain.*

LRP5 expression is detected in a variety of human tissues, with the highest level of expression in the liver hepatocytes and adrenal gland cortex (Dong et al. 1998, Hey et al. 1998, Kim et al. 1998). LRP5 is also expressed in the pancreatic beta cells and involved in the glucose-induced insulin secretion of the pancreatic islets (Figueroa et al. 2000, Fujino 2003). Looking at the expression levels in bone tissues, LRP5 is found in osteoblasts and osteocytes, but not in osteoclasts (Babij et al. 2003, Spencer et al. 2006).

4.3. LRP5 mutations and polymorphic variation in bone

LRP5 was originally discovered when searching for candidate genes causing type 1 diabetes (Hey et al. 1998). In 2001, Gong and co-workers (Gong et al. 2001) found that loss-of-function mutations in *LRP5* cause OPPG, the autosomal recessive disorder described in Section 2. Surprisingly, a year later two individual research groups reported mutations in this same gene in patients with HBM, a disorder with a bone phenotype totally opposite to that seen in OPPG (Little et al. 2002, Boyden et al. 2002). In 2004, mutations in *LRP5* were found in patients with FEVR, a disorder characterized with retinal abnormalities and low BMD (Toomes et al. 2004).

4.3.1. OPPG mutations

The reported OPPG-associated *LRP5* mutations are scattered throughout the gene with no clustering to any specific regions or domains. With only a few exceptions, they are missense mutations, affecting only one amino acid of the protein.

OPPG patients, homozygous or compound heterozygous for *LRP5* mutations, are blind and have extremely low BMD. Signal transduction assays on cell cultures have shown that these *LRP5* mutations disrupt the Wnt and Norrin signal transduction (Ai et al. 2005a). Studies on mice have revealed that disruption of *LRP5* results in a similar phenotype as seen in OPPG patients. *Lrp5*$^{-/-}$ mice have a low bone mass phenotype, which develops after birth as a secondary effect due to reduced osteoblast proliferation and function (Kato et al. 2002). These mice also undergo extensive postnatal vascularisation of the eye, causing a similar eye phenotype as seen in OPPG patients.

Interestingly, not only the OPPG patients but also the obligate mutation carriers, for example the parents of OPPG patients, have a reduced BMD (Gong et al. 2001, Lev et al. 2003). This is also seen in the mouse model, as *Lrp5*$^{+/-}$ mice have a mild delay of osteogenesis and eventually a lower BMD than their wild type (WT) littermates (Kato et al. 2002, Holmen et al. 2004). No defects in the vascularisation of the eye have been reported in these *Lrp5*$^{+/-}$ mice (Kato et al. 2002).

4.3.2. HBM mutations

All HBM mutations published to date are located in the first β-propeller domain of *LRP5*, suggesting the importance of this domain in the regulation of bone metabolism. The p.G171V mutation, reported in several HBM patients, is fairly well characterized and its role in preventing the binding of Dkk1 inhibitor protein to LRP5 has been proposed (Boyden et al. 2002). This in turn might activate Wnt/β-catenin signaling continuously and thus elevate bone density. Zhang and co-workers (2004) presented an alternative mechanism for the HBM phenotype. The p.G171V mutation was shown not to directly alter the binding of Dkk1 but to interfere with the interaction of LRP5 and its chaperone protein MESD, resulting in disruption of LRP5 transportation to the cell membrane and thereby reducing the number of targets for Dkk1 inhibition (Zhang et al. 2004).

4.3.3. LRP5 polymorphic variation

To date, almost 800 polymorphic variations have been identified in *LRP5* and 27 of these are located in the coding sequence (SNPper, http://snpper.chip.org). Many of these have shown association with BMD and other bone parameters in several individual studies (Ferrari et al. 2004, Koay et al. 2004, Mizuguchi et al. 2004, Urano et al. 2004, Koller et al. 2005, Brixen et al. 2007, Ezura et al. 2007, Giroux et al. 2007, Koay et al. 2007, Grundberg et al. 2008, Sims et al. 2008, Urano et al. 2009, van Meurs et al. 2008). Recent genome-wide association (GWA) studies and meta-analyses have further elucidated the importance of these variations on BMD (Richards et al. 2008, Tran et al. 2008, Richards et al. 2009, Rivadeneira et al. 2009, Zhang et al. 2009).

5. Human sequence variation

The phenotypic differencies seen between individuals are a result of sequence variation in their genomes. A majority of this variation is due to variation in single nucleotide polymorphisms (SNPs), whereas the rest is attributable to insertions or deletions, copy number variations (CNVs), microsatellites or other tandem repeats, and other chromosomal rearrangements (Sachidanandam et al. 2001, Stankiewicz and Lupski 2010).

The current (September 2010) version of the NCBI dbSNP database human build 131 contains over 20 million reference (rs) SNPs (http://www.ncbi.nlm.nih.gov/snp/). Recent studies on CNVs have revealed the importance of these variants on genetic variation and disease (Zhang et al. 2009). To date, almost 60 000 CNVs have been identified according to the Database of Genomic Variants (http://projects.tcag.ca/variation) and the number will probably increase as new

sequence data emerge. The challenge in most, if not all genetic research projects, is to find and identify the right pathogenic variants and mutations from this enormous amount of genetic variation. The number of reported mutations underlying or associated with human disease has exceeded 100 000 in the public databases (Cooper et al. 2010). In addition, recent GWA studies have produced over 650 publications showing association with over 3 000 SNPs and human diseases (http://www.genome.gov/gwastudies/). It is important to remember that each person carries approximately 250 to 300 loss-of-function variants in annotated genes and 50 to 100 variants previously reported in inherited disorders (The 1000 Genomes Project Consortium 2010).

5.1. Mutation types

Mutations can be divided into several groups according to their different effects on protein function.

Missense mutations are nucleotide substitutions that result in a change of the amino acid sequence. They are often difficult to distinguish from normal polymorphic variations. Pathogenic, i.e. disease-causing missense mutations, are often located in evolutionally conserved regions in functionally significant amino acid regions. They can modify the protein function by changing its chemical properties and affecting protein folding.

Nonsense mutations introduce a premature translation-termination codon that disrupts the protein synthesis. This usually leads to the degradation of the newly-formed transcript through a process called nonsense-mediated mRNA decay (NMD) (Brogna and Wen 2009). In some rare cases the transcript escapes this degradation and a truncated protein is produced (Khajavi et al. 2006). For example, if the mutation is located downstream from the last exon junction complex, the NMD-complex does not form (Nagy and Maquat 1998).

In addition to missense and nonsense mutations, a third mutation type affecting only one nucleotide is the **silent mutation**. It occurs in the third position of a codon but does not change the amino acid sequence. Silent mutations have long been thought as non-effective, but in some cases they have been shown to have an effect on RNA processing and splicing (Cooper and Mattox 1997, Chao et al. 2001).

Splicing mutations are nucleotide changes located in the conserved motifs of the exon-intron boundaries or in the branch sites inside exons (Cartegni et al. 2002). Changes in these regions may, for example, disrupt the exon splicing completely or partially, activate cryptic splice sites, or interfere with the normal splicing of splice variants.

Abnormal splicing can disrupt the reading frame and therefore cause a premature translation-termination codon which in turn leads to NMD.

Deletions, insertions and duplications of one or more nucleotides usually lead to NMD as the reading frame is disrupted and a premature translation-termination codon is produced. If the aberration length is divisible by three nucleotides, i.e. one or more codons, the reading frame is maintained and the protein might be produced normally. Large deletions, duplications, triplications, insertions, and other genomic rearrangements can all result in **CNVs** (Stankiewicz and Lupski 2010). The CNVs are partly responsible for the evolution and diversity between individuals but they can also predispose to common complex traits such as Alzheimer disease and autism (Rovelet-Recrux et al. 2006, Sebat et al. 2007).

6. Identification of disease-causing mutations and predisposing variants

6.1. Association analyses

During the past ten years, advances in microarray technology have provided cost-effective and fast ways for analysis of genetic variation. SNPs and CNVs can be analyzed at very large scales using various assays including allele-specific hybridization and DNA polymerase- and ligase-assisted genotyping (Syvänen 2005). Even the whole genome can be sequenced at a relatively robust and easily implemented manner, although the costs and computational capacity required are still significant.

In association studies, the polymorphisms of interest are usually common in the population and therefore a clear association between the phenotype and genotype cannot be made without a large scale analysis. It is estimated that a conventional SNP association study needs a sample size of 2 000 cases and 2 000 controls to give sufficient power to detect the associated common variants (Spencer et al. 2009). In the future, even whole-genome sequencing studies will be carried out with very large sample sizes as in GWA studies, but in the meantime it is important to focus on methods that are optimized to detect the role of causal genomic variants in smaller sample sizes (Cirulli and Goldstein 2010).

6.2. Mutation analyses

Mutations are often screened at a smaller scale of samples from patients, family members and controls. Probably the most common method is the direct sequencing of PCR amplified genes. Several other methods for mutation screening have also been developed, including denaturing high-performance liquid chromatography (DHPLC) (Xiao and Oefner 2001) and multiplex ligation-dependent probe amplification (MLPA)

(den Dunnen and White 2006). The most recent application for mutation screening is array-based exome sequencing (Ng et al. 2009). This method provides a cost-effective and sensitive identification of genetic variants located in the protein-coding regions of individual genomes.

When looking for disease-causing mutations it is important to distinguish them from the numerous normal polymorphic variants located in the human genome. Nonsense and splicing variants are usually mutations, as they rarely occur in healthy subjects, but missense mutations can be difficult to distinguish from SNPs. The absence of a variation from more than 200 individuals of the same ethnic group as the patient usually excludes the possibility of a common polymorphism (Antonarakis and Cooper 2001). Disease-causing mutations are usually very rare when compared to common polymorphic variants, but it is important to note that a rare variant is not always a mutation. Results from recent studies suggest that some common diseases and unfavourable traits, such as colorectal cancer and low plasma HDL cholesterol levels, are caused by a combination of multiple rare genetic variants (Bodmer and Bonilla 2008). Therefore, the analysis of control samples is mandatory in all mutation analyses.

6.3. Functional analyses

Mutation screening and identification is the first step in the process of identification of causative genes and mutations in various human disorders. To understand the molecular mechanisms of genes and their protein products and the way these are changed in the presence of a pathogenic mutation, a series of functional analyses are needed.

Web resources provide useful programs that can give helpful information when determining gene function and predicting the effects of mutations. These *in silico* programs usually provide plenty of data that can be overwhelming and sometimes even give contradictory results. Among the most useful programs are sequence alignment programs such as BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and BLAT (http://genome.ucsc.edu/chi-bin/hgBlat). These provide information about similarities between sequences in the same or different species. This can be helpful, for example, when characterizing pathogenic genetic variants, because if located in conserved regions they are more likely to be disease-causing (Mooney and Klein 2002). Moreover, web-based programs such as SIFT (http://sift.jcvi.org) and PolyPhen (http://genetics.bwh.harvard.edu/pph) are used to predict the effects of identified sequence variants. In addition, the 3D structure of proteins can be calculated with complex algorithms and this can give further clues about the protein function (Sanchez et al. 2000).

If the gene and its protein product are already characterized, functional assays in cell cultures can be useful methods. The intracellular localization of wild type and mutated proteins can be monitored in cells over-expressing the desired proteins. In addition, protein-protein interactions can be studied with numerous different assays, such as co-immunoprecipitation of protein complexes and phage display.

The disease pathogenesis and the function of pathogenic mutations can also be studied using genetically modified animal models. Mouse (*Mus musculus)* is the most commonly used model organism for studying human biology and diseases (Bult et al. 2008). Rat (*Rattus norvegicus*) is another mammalian organism commonly used, and non-mammalian models, such as round worm (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*), and zebrafish (*Danio rerio*) are also frequently used. These model organisms are genetically modified to study the biomechanical mechanisms of human diseases. The creation and maintenance of these animal models is expensive and time consuming. However, these models are valuable tools when assessing gene therapy or pharmacological therapies for patients.

AIMS OF THE STUDY

Prior to this thesis work, the *LRP5* gene had been identified and characterized as the causative gene for osteoporosis pseudoglioma syndrome (OPPG) and high bone mass disorder (HBM). A few papers, describing the association of *LRP5* polymorphisms with bone mineral density, had already been published, and during the course of the study several other *LRP5* association studies were published. The present study was aimed to identify novel *LRP5* variations and to study their association with different clinical characteristics.

The following specific aims were set for the study:

1. To determine the role of *LRP5* variation in peak bone mass (PBM) attainment.
2. To screen *LRP5* mutations in Finnish OPPG patients and their family members and to characterize their association with the clinical phenotype.
3. To determine if *LRP5* variation contributes to the susceptibility of childhood fractures.

MATERIALS AND METHODS

1. Patients and controls

1.1. Consent and ethics committee permissions

Informed written consent was obtained from all patients and healthy controls studied. The studies on Finnish army recruits, osteoporosis pseudoglioma families, and children with recurrent fractures were approved by the Research Ethics Committee of Helsinki University Hospital. The study on children with premature adrenarche was approved by the Ethics Committee of Kuopio University Hospital.

1.2. Finnish army recruits

Study I comprised 185 healthy male conscripts from the Finnish army and 50 men of the same age who had postponed their military service for reasons unrelated to health. These 235 men, aged 18.3 to 20.6 years, were part of a larger epidemiological study aimed at elucidating the role of genes, hormones, and life style factors as determinants of peak bone mass (PBM), and studying the effect of exercise on bone mass during military service.

1.3. Patients with premature adrenarche (PA)

Altogether 170 Finnish children, living in the area of the Kuopio University Hospital district, took part in Study II. Of these, 73 children (63 girls and 10 boys) had clear clinical signs of adrenarche before the age of 8 years in girls and 9 years in boys. Tumors and endocrine disorders were excluded biochemically and by abdominal ultrasound. In addition, 97 age- and gender-matched children (79 girls and 18 boys) were identified from the Finnish population register and included in the study as healthy controls.

1.4. Osteoporosis pseudoglioma patients and family members

Members of a three-generation Finnish family were included in Study III. Clinical findings for two of the family members had been described in 1988 by Somer and co-workers (Figure 4) (Somer et al. 1988). These two OPPG patients had typical clinical findings; they both were blind and had severe osteoporosis with multiple compression fractures. The causative *LRP5* mutation, homozygous p.R570W, underlying their symptoms was discovered in the first genetic study describing *LRP5* mutations (Gong *et al* 2001). In the beginning of our study, relatives of these two OPPG patients were

contacted and asked if they were willing to participate in further studies. Twenty eight family members agreed. Data on their previous medical history were collected, they were clinically assessed, blood samples were obtained for DNA and blood biochemistry, and the skeletal phenotype was determined by DXA and radiography.

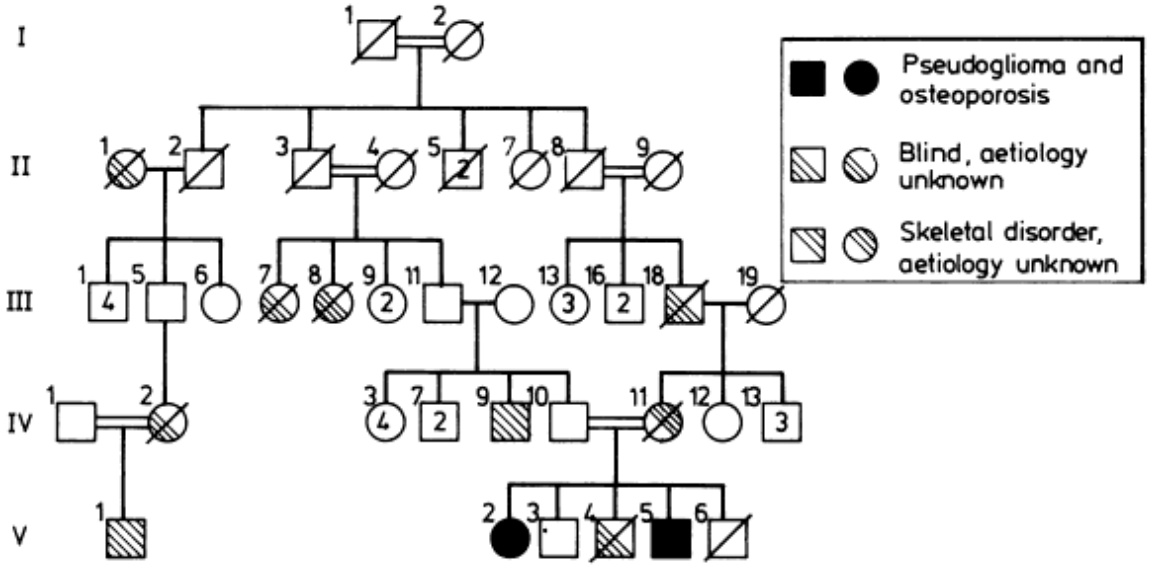

Figure 4. *A schematic pedigree showing two OPPG patients and their close family members included in the original study by Somer and co-workers (1988). Reproduced from Somer et al. (1988) with permission from the BMJ Publishing Group Ltd.*

In addition to the large Finnish family, fourteen unrelated individuals from Orton Orthopaedic Hospital (Helsinki, Finland) were screened for *LRP5* mutations as part of their osteoporosis assessment.

1.5. Children with recurrent fractures

The participants for Study IV were recruited from a cohort of children who were treated for an acute fracture at the Hospital for Children and Adolescents, University of Helsinki, during a 12-month study period in 2005-2006. For the present study all those children aged from 4 to 16 years were recruited who had experienced:

1) at least two low-energy long bone fractures before the age of 10 year, or
2) at least three low-energy long bone fractures before the age of 16 years, or
3) had sustained at least one low-energy vertebral fracture.

Children with chronic illness affecting the bone or with features suggestive of osteogenesis imperfecta (OI) or OPPG were excluded from the study. Of the 1390

children with a fracture during the study period, 72 (5.2%) fulfilled the criteria and 66 (92%) of them were willing to participate. A thorough clinical assessment and DNA analysis were performed on those children and their parents, who had a potentially significant *LRP5* variation.

1.6. Control samples

The controls used in Studies III and IV were unrelated Finnish Caucasian samples from the Finnish Red Cross Blood Transfusion Service and other unrelated healthy subjects of Finnish origin.

2. Methods

The clinical, radiological, and biochemical studies performed to determine the phenotype of the study subjects are summarized in Table 2 and described in detail in the original publications I-IV. The methods used in the genetic studies are summarized in Table 3.

Table 2. *Clinical, radiological, and biochemical studies performed in this thesis.*

Method	Original publication
Clinical assessment and biochemistry	I, II, III, IV
DNA analysis	I, II, III, IV
Glucose metabolism	II, III
Lipid profile	II, III
Radiography, ultrasound, and bone densitometry	I, III, IV
Opthalmology	III

2.1. Clinical assessment and biochemistry (I, II, III, IV)

The study subjects were clinically assessed (Studies II, III and IV); anthropometry (Studies I, II, III, and IV) and pubertal stage according to Tanner (Studies II and IV) were determined. Blood and urine samples were obtained for parameters of calcium homeostasis and bone turnover markers, including serum/plasma calcium (Study IV), phosphate (Study IV), alkaline phosphatase (Study IV), 25-hydroxyvitamin D (25-OHD) (Studies I and IV), parathyroid hormone (PTH) (Studies I and IV), type I procollagen aminoterminal propeptide (PINP) (Studies I and III), total osteocalcin (TOC) (Study I), carboxylated osteocalcin (COC) (Study I), tartrate-resistant acid phosphatase 5b (TRACP5b) (Study I), and urinary type I collagen aminoterminal telopeptide (NTX) (Studies I and III). Serum cortisol, dehydroepiandrosterone sulphate (DHEAS),

dehydroepiandrosterone (DHEA), androstenedione, and sex hormone binding globulin (SHBG) were used to assess adrenal function (Study II). Serotonin (5-hydroxytryptamine, 5-HT) was determined in subjects participating in Study III.

2.2. Glucose metabolism and lipid profile measurements (II, III)

Plasma glucose, serum insulin, and C-peptide concentrations were measured to evaluate glucose tolerance and insulin secretion. An oral glucose tolerance test (OGTT) was performed after an overnight fast. For the evaluation of insulin sensitivity, homeostasis model assessment for insulin resistance (HOMA-IR) was calculated. Specific methods and reference values are described in the original publications (II and III).

Total plasma cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol and triglycerides were analyzed after an overnight fast with enzymatic methods. Specific methods and reference values are described in the original publications (II and III).

2.3. Radiography, ultrasound, and BMD measurements (I, III, IV)

Anterior-posterior and lateral radiographs of the thoracic and lumbar spine were obtained to identify vertebral compression fractures. X-ray examination was used to confirm stress fractures in Study I, and fractures and bone age in Study IV. In Study I, stiffness of the bone was calculated using broadband ultrasound attenuation (BUA) and speed of sound (SOS) measurements of the heel. BMC and BMD for the lumbar spine, femoral neck, proximal femur, and whole body were measured with dual-energy X-ray absorptiometry (DXA) (Studies I, III, and IV).

2.4. Opthalmological measurements (III)

Study subjects with heterozygous *LRP5* mutations (Study III) were examined for visual acuity, refraction, and ocular abnormalities. Vitreous and retina were evaluated after papillary dilatation and abnormal findings were recorded with a Panoret 1000 wide-angle digital fundus camera (Medibell Medical Vision Technologies Ltd, Valley Stream, NY, USA).

Table 3. Methods used in the genetic studies of this thesis.

Method	Original publication
Agarose gel electrophoresis	I, II, III, IV, U
Cell culture	III, U
DNA isolation and purification	III, IV, U
DNA sequencing	I, II, III, IV, U
Immunofluorescence microscopy	U
Immunofluorescence staining	U
In silico sequence analysis	I, II, III, IV, U
Multiplex ligation-dependent probe amplification (MLPA)	U
Polymerase chain reaction (PCR)	I, II, III, IV, U
Recombinant DNA techniques (cloning)	III, U
Restriction analysis	III, U
Site-directed mutagenesis	III, U
TaqMan SNP genotyping	I
Transient transfections	III, U
Wnt/β-catenin signaling assays	III

2.5. DNA isolation and purification (III, IV, U)

Genomic DNA was extracted according to manufacturer's instructions from peripheral blood (Puregene DNA Purification kit, Gentra Systems, Minneapolis, MN, USA) or saliva (Oragene, DNA Genotek Inc., Ontario, Canada). DNA concentration and purity were measured using a standard spectrophotometer or NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Amplification and sequencing (I, II, III, IV, U)

Genomic DNA was amplified via polymerase chain reaction (PCR) with standard methods. PCR and sequencing primers were generated using Primer3 (http://frodo.wi.mit.edu/primer3), Repeat Masker (http://www.repeatmasker.org), and UCSC Blat (http://genome.ucsc.edu/). Primers were designed to amplify all 23 exons and exon-intron boundaries and flanking 3' and 5'UTR regions of the *LRP5* gene. PCR products were purified enzymatically using ExoSAP-IT (USB, Cleveland, OH, USA), labeled with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and then sequenced with ABI 3730 DNA analyzer (Applied Biosystems).

2.7. Sequence analysis and predictions (I, II, III, IV, U)

Sequences were analyzed using BioEdit (Ibis Therapeutics, Carlsbad, CA, USA) and Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA). Web-based tools Sift

(http://sift.jcvi.org), PolyPhen (http://genetics.bwh.harvard.edu/pph), and ESEfinder 2.0 (http://rulai.cshl.edu/tools/ESE2) were used to predict the effects of identified *LRP5* sequence variants.

2.8. Restriction analysis (III, U)

The identified OPPG mutations p.R570W and p.R1036Q were screened in family members and controls using restriction enzyme digestion of PCR-amplified DNA. Exons 8 and 14, respectively, were amplified from genomic DNA and then digested using BsrBI (for p.R570W) or HpaII (for p.R1036Q) restriction enzymes (New England Biolabs, Ipswich, MA, USA). Digested PCR products were run on a 2% agarose gel and visualized with ethidium bromide under UV light.

2.9. TaqMan SNP genotyping assays (I)

TaqMan SNP genotyping assay was used to detect three *LRP5* polymorphisms: p.Q89R, p.F549F, and p.V667M. Samples in a 384-well PCR plate were amplified with specific TaqMan primers and probes provided by Applied Biosystems. SNP detection and allelic discrimination was performed using ABI Prism 7900HT Sequence Detection System (SDS, Applied Biosystems).

2.10. Cell lines (III, U)

Commercially available cell lines of human cervical cancer cells (HeLa), human embryonic kidney cells (HEK-293T), and African green monkey kidney cells (COS-1) from the American Type Culture Collection (ATCC) were used in the *Wnt*/β-catenin signaling assays (III) and immunofluorescent LRP5 trafficking assays (U).

2.11. Mutagenesis and transfections (III, U)

The wild type (WT) and/or mutant vectors were introduced to HeLa, HEK-293T, and COS-1 cells using FuGENE 6 (Roche Diagnostics, Mannheim, Germany) or Lipofectamine LTX PLUS (Invitrogen, Carlsbad, CA, USA) transfection reagents.

For mutagenesis, QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used to introduce identified sequence variants into the vector. The pcDNA3.1(-) expression vector, containing the full length WT human *LRP5* cDNA, was kindly provided by Dr. Matthew Warman. In order to verify correct changes, inserts were sequenced with specific exonic primers (primer design and sequencing were performed as described previously).

2.12. Wnt/beta-catenin signaling assays (III)

In order to study the role of the identified sequence variants on LRP5 signal transduction we performed a series of *Wnt*/β-catenin signaling assays, described earlier by Ai and co-workers in 2005. Expression constructs Wnt1-v5, Topflash, pRL-TK, and MESD-C2, together with WT and/or mutant *LRP5* in pcDNA3.1 were transfected into HEK293T cells with FuGENE 6. If needed, β-galactosidase (LacZ) in pcDNA3.1 was used to ensure equal amounts of DNA in each transfection. After 30 hours, cells were lysed and Firefly (from Topflash) and Renilla (from pRL-TK) luciferase activities were measured using the Dual Luciferace Assay Kit (Promega, Madison, WI, USA) and Wallac Victor 1420 luminometer (Perkin Elmer, Waltham, MA, USA).

The WT *LRP5* in pcDNA3.1 and the expression constructs Wnt1-v5, Topflash, pRL-TK, and MESD-C2 were all kindly provided by Dr. Matthew Warman and his co-workers.

2.13. Immunofluorescence staining and visualization of transfected cells (U)

In order to study the role of the identified sequence variants on LRP5 localization and transport to the cell membrane, we performed a series of immunofluorescent assays. HeLa, HEK-293T, and COS-1 cells were transfected with WT or mutant Myc-tagged *LRP5* in pcDNA3.1, MESD-C2, and RAP expression constructs using FuGENE 6 or Lipofectamine LTX with PLUS reagent. Transfection efficiency was tested using a GFP-tagged Cystatin B expression construct. Confluent cells growing in cover slips were treated with cyclohexamide 2 hours prior to fixation to stop the biosynthesis of proteins. Fixation of the cells was performed using 4% paraformaldehyde (PFA) or -20°C methanol (MeOH). PFA fixed cells were permeabilized with 0.1% Triton-X-100 in phosphate buffered saline (PBS). Cells were then blocked with 0.2% bovine serum albumin (BSA) or 10% fetal calf serum (FCS) in PBS. Stainings were performed using the following primary and secondary antibodies: mouse anti-c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-pan-Cadherin (Zymed laboratories, San Francisco, CA, USA), Alexa Fluor® 594 donkey anti-mouse (Invitrogen), and Alexa Fluor® 488 donkey anti-rabbit (Invitrogen). Nuclear staining was performed using DAPI or Hoechst. Stained cells were visualized with a Zeiss Axioplan 2 microscope and digital camera, and Axiovision 3.1 software.

2.14. Multiplex ligation-dependent probe amplification (MLPA) (U)

We used multiplex ligation-dependent probe amplification (MLPA) to look for possible exonic amplifications and deletions in DNA samples of the 66 children with recurrent fractures (Study IV). The Salsa MLPA P285-A1 LRP5 probemix (MRC-Holland, Amsterdam, The Netherlands) contained 38 specific probes for each of the 23 exons for

LRP5 and each of the 4 exons of *DKK1*. Furthermore, an additional probe for the *LRP5* p.G171V HBM mutation, located in exon 3, was included. Each probe had a unique "stuffer" sequence of variable length so that they could be distinguished from each other using electrophoresis.

Denaturated genomic DNA was first hybridized with the probe mix for 16-18 hours. Hybridized probe pairs were then ligated and amplified via PCR. PCR products, with sizes ranging from 136 to 436 nucleotides, were then separated with an ABI 3730 DNA Analyzer (Applied Biosystems) and fragment sizes were determined using GeneMapper v4.0 (Applied Biosystems). The eventual MLPA data was normalized and analyzed using Coffalyser software (MRC-Holland).

2.15. Statistical analyses (I, II, III, IV)

Statistical analyses were performed using the Statistical Package for Social Sciences for Windows 14.0 or 17.0 (SPSS Inc., Chicago, IL, USA) and SAS® System version 8.02 for Windows (SAS Institute Inc., Cary, NC, USA). Fisher's exact tests, Student's T-tests, and Mann Whitney U tests were used to test differences between genotype and phenotype groups, as appropriate. In Study I, the association analyses were performed with a one-way analysis of variance (unadjusted analysis) and a multiple regression model using life style factors (age, height, weight, smoking, exercise, alcohol, and calcium intake) as adjusting factors. A *p*-value less than 0.05 was considered statistically significant. Chi-square (χ^2) tests were used to calculate Hardy-Weinberg equilibrium. Linkage disequilibrium (LD) was analyzed with HaploView 3.2 or 4.0.

RESULTS AND DISCUSSION

All the *LRP5* variants found during the course of this thesis are listed in Table 4. The corresponding locations of the exonic variants in the LRP5 protein domains are presented in Figure 5. The characterization and role of these variants in different clinical entities and the relevance of these results in a larger context will be discussed in detail in the following sections.

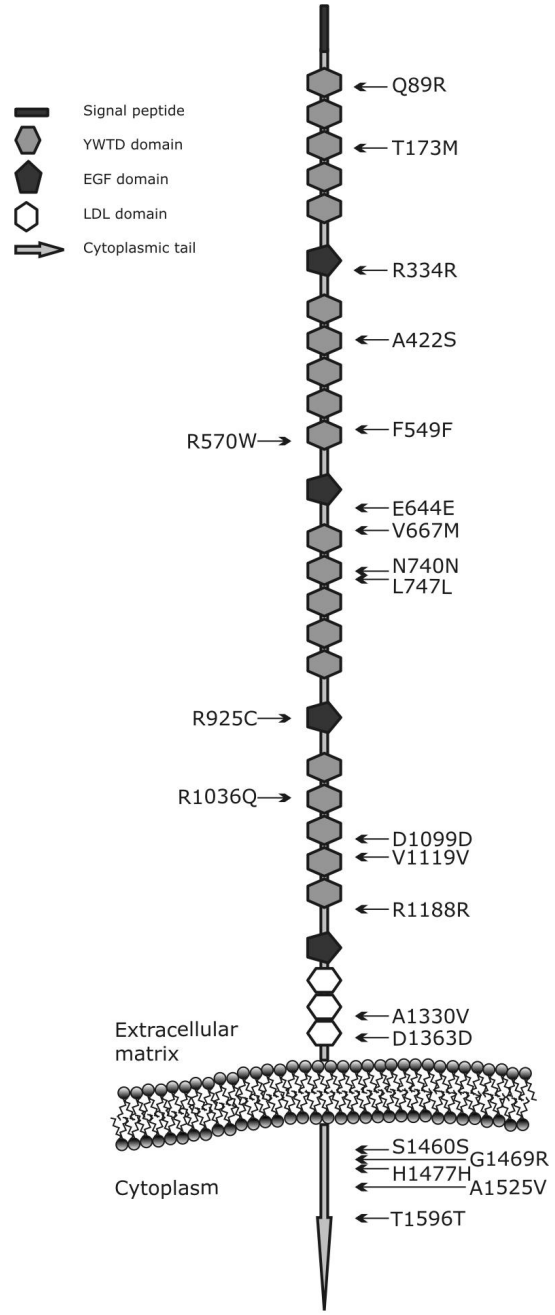

Figure 5. *A schematic drawing of the LRP5 protein and the location of all exonic variants found in Studies I-IV. The polymorphic variants are depicted on the right side of the protein and the three OPPG related LRP5 missense mutations are marked on the left of the protein structure.*

1. Genetic variation in *LRP5* and skeletal characteristics

The low BMD seen in OPPG patients and the high bone mass phenotype seen in HBM patients are due to mutations in the *LRP5* gene (Gong et al. 2001, Little et al. 2002, Boyden et al. 2002). In addition, several common polymorphisms in the *LRP5* gene have been associated with low BMD, suggesting an important role for this gene in normal bone development. Moreover, studies on *LRP5* deficient mice ($Lrp5^{-/-}$) have further demonstrated that LRP5 and its homologue LRP6 have important roles in bone development (Holmen et al. 2004). The biological mechanism causing the abnormal bone phenotype in these mice and in human patients is thought to be due to the reduction of signal transduction of the Wnt/β-catenin signaling pathway (Ai et al. 2005a), although alternative pathways have also been suggested (Yadav and Ducy 2010). Encouraged by these previous studies, we decided to examine the role of *LRP5* genetic variation on different clinical bone characteristics in several Finnish cohorts.

1.1. Polymorphic LRP5 variations are associated with low peak bone mass (I)

The influence of common *LRP5* polymorphisms on normal variation in BMD was demonstrated quite soon after the original discovery of OPPG associated mutations in *LRP5* (Ferrari et al. 2004, Koay et al. 2004, Mizuguchi et al. 2004, Urano et al. 2004, Bollerslev et al. 2005, Zhang et al. 2005). These studies were mainly performed using samples from post-menopausal women, although in some cases samples from men were also used (Ferrari et al. 2004, Koh et al. 2004). Nevertheless, the role of these variants on bone mass accrual and development of peak bone mass (PBM) in early adolescence remained unclear.

In our study we searched for exonic *LRP5* variants in DNA samples from 235 Finnish men aged 18 to 20 years, i.e. the age when BMD is the highest (PBM). These men underwent a series of clinical assessments including bone mineral density measurement by DXA. The aim of this study was to identify *LRP5* variants that would associate with low BMD. Since PBM is an important determinant of BMD later in life, the identification of these variants would be important.

For the analysis, we selected 10 polymorphic *LRP5* variants that had been associated with variation in BMD in previous studies (Van Wesenbeeck et al. 2003, Ferrari et al. 2004, Koay et al. 2004, Mizuguchi et al. 2004, Ferrari et al. 2005, Koller et al. 2005, Zhang et al. 2005, van Meurs et al. 2006). These variants were spread throughout the gene; eight were located in exons and two were in introns in the immediate vicinity of the exon-intron boundaries. One of the alterations, p.Q89R in exon 2, was not found in any of the study subjects. However, the remaining nine variants were identified and

Table 4. *Summary of all the LRP5 variants found in Studies I-IV. The three putative mutations described in Study III are marked in bold.*

Exon/ Intron	Base change	Amino acid change	Thesis study	Previously associated with
Exon 2	c.266A>G	p.Q89R	II	BMD[1-3] hypertension[4] osteoarthritis[5]
Exon 3	c.518C>T	p.T173M	IV	FEVR[6]
Intron 4	c.844-4C>T		II	
Exon 5	c.1002G>A	p.R334R	IV	
Exon 6	c.1264G>T	p.A422S	IV	
Intron 6	c.1412+8G>A		I, II	
Exon 8	c.1647C>T	p.F549F	I, II, IV	BMD[7]
Exon 8	**c.1708C>T**	**p.R570W**	**III**	**OPPG[8]**
Exon 9	c.1932G>A	p.E644E	I, II, IV	BMD[7]
Exon 9	c.1999G>A	p.V667M	I, II, IV	BMC/BMD[8-10] idiopathic osteoporosis[11] PBM[12]
Exon 10	c.2220C>T	p.N740N	I, II, IV	BMD[2,3,13,14]
Exon 10	c.2241G>A	p.L747L	IV	
Intron 11	c.2318+6T>C		I, II	
Exon 12	**c.2773C>T**	**p.R925C**	**III**	
Exon 14	**c.3107G>A**	**p.R1036Q**	**III**	**juvenile osteoporosis[15]**
Exon 15	c.3297C>T	p.D1099D	I, IV	
Exon 15	c.3357C>T	p.V1119V	I, II, IV	BMC/BMD[14,16] osteoporotic fractures[16]
Exon 16	c.3564G>A	p.R1188R	II, IV	
Exon 17	c.3723A>G	p.P1241P	II	
Exon 18	c.3989C>T	p.A1330V	I, II, IV	BMD[9,13,17-21] blood pressure[22] C-peptide and proinsulin levels[23] hypercholesterolaemia[24] idiopathic osteoporosis[11] PBM[12]

Exon 19	c.4089C>T	p.D1363D	II	
Exon 21	c.4380C>T	p.S1460S	II, IV	
Exon 21	c.4405G>A	p.G1469R	II, IV	
Exon 21	c.4431C>T	p.H1477H	II	
Exon 22	c.4574C>T	p.A1525V	II, IV	osteoblast differentiation[25]
Exon 23	c.4788C>T	p.T1596T	II	
3'UTR	ex23+4C>T		II	

BMC, bone mineral content; BMD, bone mineral density; FEVR, familial exudative vitreoretinopathy; PBM, peak bone mass; SNP, single nucleotide polymorphism. Mutations and polymorphic SNPs are numbered using the nucleotide (NM_002335) and protein (NP_002326) sequences according to the official Mutation Nomenclature Guidelines (http://www.hgvs.org/mutnomen). Referred publications: 1) Koh et al. 2004, 2) Zhang et al. 2005, 3) Lau et al. 2006, 4) Suwazono et al. 2000b), 5) Urano et al. 2007, 6) Toomes et al. 2004, 7) Utriainen et al. 2009, 8) Gong et al. 2001, 9) Giroux et al. 2007, 10) Grundberg et al. 2008, 11) Ferrari et al. 2005, 12) Brixen et al. 2007, 13) Mizuguchi et al. 2004, 14) Koay et al. 2007, 15) Hartikka et al. 2005, 16) Bollerslev et al. 2005, 17) van Meurs et al. 2006, 18) Ezura et al. 2007, 19) Kruk et al. 2009, 20) Lee et al. 2009, 21) Urano et al. 2009, 22) Suwazono et al. 2006c, 23) Bendlova et al. 2008, 24) Suwazono et al. 2000a, 25) Guo et al. 2007.

the allelic distributions in the study subjects were in line with those expected under Hardy-Weinberg equilibrium.

Combining the genetic and clinical data revealed significant association between the p.A1330V variant and several bone parameters (Table 5). Individuals with the p.A1330V variant (n=20) had significantly lower BMD and BMC values in the femoral neck, trochanter, and lumbar spine compared to individuals without p.A1330V (n=215). In addition, the total hip BMD and BMC and bone strength and elasticity, measured from the heel, were significantly lower in those with the p.A1330V variant. No significant association with bone parameters was seen with the remaining eight *LRP5* variants.

The p.A1330V variant is located in exon 18, a region encoding the second LDL-domain of the LRP5 protein. Although the function of this domain is unclear, some predictions can be made according to its homology with other LDL domains in other proteins. For example, similar LDL domains in the LDL receptor are involved in the binding of lipoprotein particles (Hey et al 1998, Gent and Braakman 2004). The LDL receptor regulates blood cholesterol levels through this lipoprotein uptake, and mutations in the *LDLR* gene cause familial hypercholesterolaemia (Gent and Braakman 2004). The

results from our study and from other subsequent studies show that the p.A1330V variant in *LRP5* also associates with low BMD, indicating that this variant might alter LRP5 signal transduction, possibly by decreasing the binding capacity of extra-cellular Wnt ligands or inhibitory proteins.

Table 5. *Skeletal findings in the study subjects with and without the A1330V variant. Mean values after adjusting for life style factors (age, weight, height, exercise, smoking, calcium and alcohol intake) are shown.*

	Ala/Ala (n=215)	***Ala/Val (n=20)***	***P***
Lumbar spine BMC (g)	61.2	58.1	0.10
Lumbar spine scan area (cm^2)	49.2	48.6	0.52
Lumbar spine BMD (g/cm^2)	1.241	1.185	**0.047**
Femoral neck BMC (g)	6.47	6.07	**0.043**
Femoral neck scan area (cm^2)	5.44	5.49	0.51
Femoral neck BMD (g/cm^2)	1.189	1.104	**0.0082**
Trochanter BMC (g)	14.8	13.2	**0.014**
Trochanter BMD (g/cm^2)	0.984	0.912	**0.016**
Total hip BMC (g)	42.9	39.4	**0.0068**
Total hip BMD (g/cm^2)	1.186	1.104	**0.0089**
BUA (dB/MHz)	79.5	75.4	0.23
SOS (m/s)	1560	1547	**0.035**

BMC; bone mineral content; BMD, bone mineral density; BUA, broadband ultrasound attenuation; SOS, speed of sound.

Our study consisted of a relatively small number (n=235) of samples, which may have prevented us from observing associations between some of the variants and bone mass. As all the *LRP5* variants tested in this study had shown association with BMD in previous studies, the fact that only one of these variants showed a significant association with low BMD in our study was probably due to the small sample size or cohort characteristics as many of the previous studies have assessed only elderly subjects and mostly women. However, our finding is in line with results from several other studies, confirming the association between the p.A1330V variant and low BMD (Van Wesenbeeck et al. 2003, Ferrari et al. 2004, Koay et al. 2004, Mizuguchi et al. 2004, Ferrari et al. 2005, Koller et al. 2005, Zhang et al. 2005, van Meurs et al. 2006, Giroux et al. 2007, Urano et al. 2009). For example, in a cohort of 6373 men and women, aged over 55 years, the p.A1330V variant associated with decreased BMD at the lumbar spine and femoral neck (van Meurs et al. 2006). Interestingly, the association was more prominent in men than in women. Furthermore, a large study (n=5144) on pre- and post-menopausal women 25 to 91 years of age showed a clear association between low BMD and the p.A1330V and p.V667M variants (Giroux et al. 2007). Interestingly, the BMD values were even lower in individuals with both of these

variants. In another study the p.V667M variant, but not p.A1330V, was associated with various bone characteristics (Ferrari et al. 2004). This study was performed in a cohort of 164 men and 200 women under 57 years, and thus it is likely that the association with p.A1330V remained unclear because of the small study sample size.

As studies with small sample size often have insufficient statistical power to detect associations, meta-analyses provide more statistical power by pooling the results from different studies. The results from our study were used in a comparative meta-analysis that included data from seven different publications concerning the association between the *LRP5* p.A1330V variant and BMD (Lee et al. 2009). The results showed a clear association between p.A1330V and low BMD. Finally, data from three independent meta-analyses and large scale GWA studies highlighted p.A1330V, among many other alterations, as an important genomic variant contributing to BMD (Tran et al. 2008, Richards et al. 2009, Zhang et al. 2009).

To conclude, our study of 235 Finnish young men showed an association between the p.A1330V variant in *LRP5* and low BMD. This is parallel to the results seen in several other association studies. Most importantly, our results indicate the importance of LRP5 in the acquisition of PBM, a determinant of BMD in later life.

1.2. The role of LRP5 variation in childhood fractures (IV, U)

Low BMD and previous fractures can predispose to new fractures. Although fractures are very common in children, recurrent fractures may be a sign of primary or secondary osteoporosis. Primary forms of osteoporosis are rare in children and usually caused by heritable disorders affecting the connective tissue. The most common of these is osteogenesis imperfecta (OI). A common polymorphic variation in the *LRP5* gene has been shown to associate with osteoporotic fractures in men and women aged over 60 years (Richards et al. 2008). In addition, heterozygous mutations in *LRP5* have been found in three children with primary osteoporosis, characterized by reduced BMD and/or increased tendency to fracture (Hartikka et al. 2005). These results inspired us to study the role of common polymorphic *LRP5* variants in childhood fractures. Our aim was to study whether these variants were over-represented in a cohort of 66 Finnish children with recurrent fractures.

Clinical assessments and data from questionnaires revealed that these 66 children had sustained altogether over two hundred fractures ranging from one spinal compression fracture to seven long bone fractures (Table 6). Spinal radiographs showed compression fractures in eleven patients. Sequencing of the exons and exon-intron boundaries of *LRP5* revealed 15 exonic variants in 22 (22/66; 33%) children (Table 4). Intronic variants were found in most of the samples but these were not included in

further studies. Of the 15 exonic variants, seven were regarded as common polymorphisms and were considered non-significant. The remaining eight variants were rare (seen in less than 1.5% of the control samples) and were therefore included in the final study. Four of these variants (p.T173M, p.A422S, p.G1469R, and p.A1525V) resulted in an amino acid substitution and four (p.R334R, p.L747L, p.R1188R, and p.S1460S) were silent changes. According to the SIFT and PolyPhen programs, only one of the missense variants (p.G1469R) was predicted to be possibly damaging to protein function.

Table 6. *Clinical characteristics of the 66 fracture prone children.*

Characteristic	
N	66
Age, mean ±SD (years)	10.7 ± 3.0
Sex, males	45 (68%)
Height (Z-score), mean SD	0.7 ± 1.0
Height adjusted weight (%), mean SD	0.84 ± 1.7
BMI (kg/m^2)	18.9 ± 3.3
BMD (Z-score)	
Lumbar spine	-0.5 ± 1.0
Femoral neck	-0.1 ± 0.9
Whole body	-0.1 ± 0.9
Fractures, no of patients	
Low-energy vertebral fracture	11 (17%)
Clavicle fractures	17 (26%)
Upper limb fractures	58 (89%)
Lower limb fractures	19 (29%)
0-1 fractures	9 (14%)
2-3 fractures	40 (61%)
4 or more fractures	17 (26%)

BMD, bone mineral density; BMI, body mass index.

The above-mentioned eight *LRP5* variants were identified in nine children (Table 7). These children and the remaining 57 children with recurrent fractures did not differ in age, sex, or number of fractures. However, clavicle fractures were more prevalent in this group (56% vs. 21%). A tendency towards younger age at first fracture (median 2.0 years vs. 5.0 years) was also seen. Despite the high number of fractures, the DXA measurements showed no clear signs of reduced BMD in these children. Instead, most (8/9) had BMD Z-scores within the normal range. This indicates that the fractures might be due to impaired bone quality, not quantity.

Table 7. *Clinical characteristics of the 9 fracture prone children with rare LRP5 variants.*

Patient no	Amino acid change	Sex	Age	Lumbar spine BMD (Z-score)	No of fractures
#1	p.T173M	M	15	-0.4	3
#2	p.A422S	F	6	-2.8	2
#3	p.R1188R	F	9	-1.3	2
#4	p.S1460S	M	6	-0.1	7
#5	p.S1460S	M	13	-0.5	4
#6	p.G1469R	M	15	-0.2	4
#7	p.G1469R	M	8	-1.1	2
#8	p.A1525V	F	7	-1.0	2
#9	p.R334R and p.L747L	M	7	+0.9	2

BMD, bone mineral density; F, female; M, male.

The role of these eight variants on BMD and susceptibility to fractures was further studied within the individual families. Parents from 8/9 families were willing to participate in these studies. Clinical assessments of the parents revealed no clear correlation between the genotypes and the phenotypes. Contrary what had been expected, the BMD values in some families seemed to be even higher in those parents with the corresponding variant.

Although the number of children with fractures in the beginning of this study was relatively high (n=1390), the number of children with recurrent fractures who fulfilled the inclusion criteria (n=72; 5.2%) and were willing to participate in the study was low (n=66; 4.7%). Therefore, the association analyses with common *LRP5* variants did not have enough power to reach statistical significance. However, the p.V667M, p.N740N, and p.A1330V variants seemed to be slightly more common in patients than in controls (4.5% vs. 2.6%, 10.6% vs. 7.2%, and 10.6% vs. 8.5%, respectively). Instead, we concentrated our studies on the rare variants. Comparison of the clinical data between fracture prone children with and without the rare *LRP5* variants and results from the genotype-phenotype assessments of individual families let us conclude that the rare *LRP5* variants identified in this study are unlikely to be the definite cause of fractures in these children. However, the specific role of these variants remained unclear and they may, together with other genetic or environmental factors, predispose to fractures. It is not unreasonable to believe that the predisposition to fractures comes through a combination of several predisposing SNPs, rather than through just one SNP alone. Interestingly, studies on *Lrp5* and *Lrp6* deficient mice have shown that introduction of an inactivating mutation to *Lrp6* further reduces BMD in the *Lrp5* deficient mice, suggesting an overlapping role of these genes during bone mass accrual (Holmen et al. 2004).

1.2.1. MLPA analysis (U)

To date, only a few studies have reported intragenic exon-spanning deletions within the *LRP5* gene (Chung et al. 2009, Narumi et al. 2010). However, this could be due only to the commonly used PCR-based mutation detection methods with genomic DNA, as these usually cannot detect exonic deletions. As RT-PCR and MLPA-based methods become more commonly used the characterization of novel *LRP5* exonic deletions is likely to occur. Consequently, it has been estimated that intragenic deletions would account approximately 5% of the *LRP5* mutations (Chung et al. 2009). As sequencing of the *LRP5* gene did not reveal any clear mutations in the 66 children with recurrent fractures, we decided to search for possible exonic deletions or amplifications in *LRP5* using MLPA.

Analyses performed with a commercially available MLPA kit, containing probes for exons in *LRP5* and *DKK1*, did not detect any exonic deletions. However, in nine samples a possible duplication of *LRP5* exon 18 was detected. Several PCR primer pairs were generated to detect and characterize this in genomic DNA. Unfortunately, these attempts failed and we could not confirm the existence of these aberrations. As RNA samples were not available from the studied children, we could not use RT-PCR-based methods. Another method, such as the high-density oligonucleotide-based array comparative genomic hybridization (aCGH) assay used in the work by Narumi and co-workers (2010), would be needed to verify and characterize the possible duplications.

In conclusion, mutations and exonic deletions in *LRP5* are not frequent alterations underlying recurrent fractures in children. Further studies in larger cohorts and extended pedigrees are needed to evaluate the effect of common and rare *LRP5* variants on childhood fractures. The role and existence of exonic amplifications in *LRP5* will need to be verified in further studies.

1.3. LRP5 mutations in the Finnish OPPG family (III, U)

The first publication showing the association between OPPG and the *LRP5* gene described mutations in 17 OPPG patients in 12 families (Gong et al. 2001). One of these families was of Finnish origin and included two siblings with OPPG. The putative *LRP5* mutation in these patients was found to be a homozygous C to T transition (c.1708C>T) in exon 8, changing the corresponding amino acid from arginine to tryptophan (p.R570W). The original study by Somer and co-workers (1988) described the clinical features of these two patients but also stated that several other family members showed signs of bone and eye involvement. The finding of the disease-causing *LRP5* mutation in this family encouraged us to contact more family members in the hope of

finding previously unknown OPPG patients and the obligate OPPG mutation carriers and to assess the clinical phenotype of these individuals more thoroughly.

The two OPPG patients and 28 of their close relatives were willing to participate in further genetic and clinical studies. One additional individual with severe primary osteoporosis and an identified *LRP5* mutation was invited to participate. Sequencing of the 23 exons with corresponding exon-intron boundaries and the flanking 5' and 3' UTR regions of *LRP5* revealed two different missense mutations in the OPPG family (Figure 6). The previously reported p.R570W mutation was found in nine individuals; in a homozygous state in the sib pair with the OPPG diagnosis and in a heterozygous state in seven family members. Surprisingly, another missense mutation (c.3107G>A, p.R1036Q) located in exon 14 was found in a heterozygous state in four individuals; one of these was a compound heterozygote for p.R570W and p.R1036Q. No *LRP5* mutations were found in the remaining eighteen family members. The additional individual with severe osteoporosis had a novel heterozygous missense mutation (c.2773C>T, p.R925C) located in exon 12.

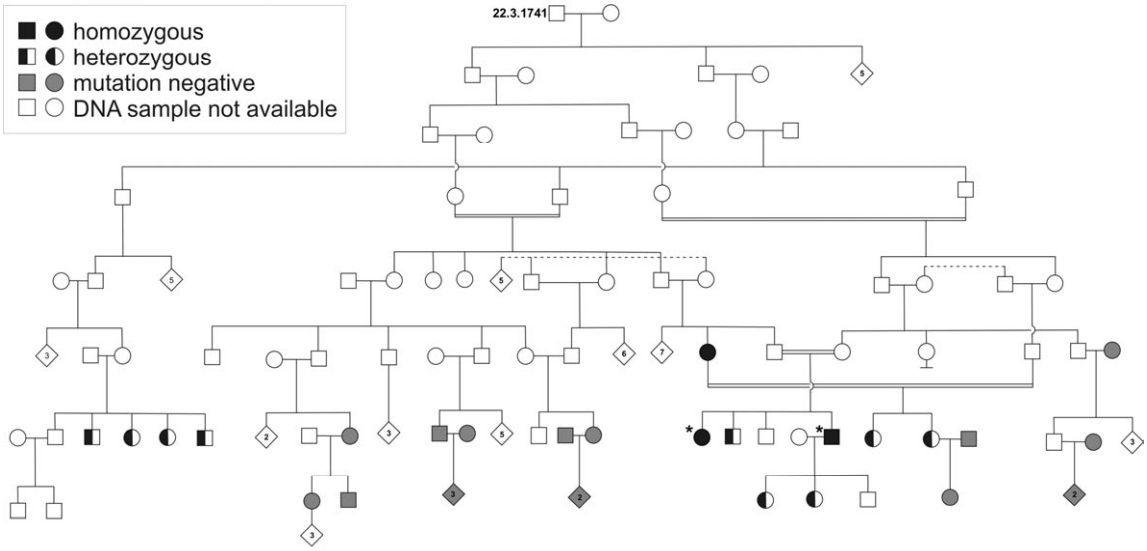

Figure 6. *The large nine-generation Finnish family with OPPG-associated LRP5 mutations. The individuals participating in the current study and the corresponding genotypes (homozygous vs, heterozygous) are marked. The two original OPPG patients homozygous for R570W are indicated with an asterisk.*

The two previously diagnosed OPPG patients with a homozygous p.R570W mutation had a typical OPPG phenotype. Both patients had severe osteoporosis with multiple compression fractures and they had both been blind since early childhood. The skeletal measurements showed clear signs of osteoporosis (Table 8). Their BMD T-score values were -5.4 and -3.3 in the lumbar spine and -5.8 and -2.1 in the femoral neck,

respectively. No accurate BMD measurements were available for the compound heterozygote for p.R570W and p.R1036Q. However, clinical records showed that she had severe osteoporosis and multiple compression fractures. Interestingly, she did not have the typical eye phenotype seen in OPPG patients but had normal vision.

Identification of the ten heterozygous *LRP5* mutation carriers gave us a unique opportunity to perform a systematic analysis of the bone phenotype in *LRP5* mutation carriers (Table 8). Our studies revealed that *LRP5* mutations, even in a heterozygous state, have an effect on BMD. Moreover, we were the first group to demonstrate the high number of spinal compression fractures in these mutation carriers. Bone density measurements revealed that the mean BMD T-score and Z-score values in mutation carriers (lumbar spine -2.4 and -0.9, femoral neck -2.1 and -0.8) were significantly lower than those seen in the mutation negative individuals (lumbar spine -0.2 and +0.4, femoral neck -0.3 and +0.8). In addition, spinal compression fractures were more common in mutation carriers (7/9; 78%) than in the individuals with no mutations (3/18; 17%).

Table 8. *Skeletal findings in patients with LRP5 mutations.*

Patient				Fractures		BMD (lumbar spine)	
no	**Age**	**Sex**	**Genotype**	**Peripheral**	**Spinal**	**T-score**	**Z-score**
#1	58	F	R570W / R570W	>3	Multiple	-5.4	-4.1
#2	52	M	R570W / R570W	2	Multiple	-3.3	-2.9
#3	15	F	R570W / WT	1	No	-0.3	-0.3
#4	19	F	R570W / WT	0	No	-0.7	-0.7
#5	72	F	R570W / WT	1	Multiple	-4.3	-2.1
#6	67	F	R570W / WT	3	Multiple	-2.7	-0.7
#7	60	M	R570W / WT	5	Multiple	-2.5	-1.8
#8	57	M	R570W / WT	3	One	-2.2	-1.7
#9	58	M	R1036Q / WT	0	One	-1.5	-1.0
#10	59	F	R1036Q / WT	1	One	-3.4	-2.0
#11	60	F	R1036Q / WT	0	NA	-1.4	+0.1
#12	85	F	R570W / R1036Q	4	Multiple	NA	NA
#13	69	F	R925C / WT	2	Multiple	-2.0	+0.2

BMD, bone mineral density; F, female; M, male; NA, data not available; WT, wild type

The results from our study with heterozygous *LRP5* mutation carriers are concordant with those seen in previous studies. The significance of the *LRP5* mutations in the OPPG mutation carriers was already noticed in the publication of Gong and co-workers (2001). They reported that OPPG mutation carriers had reduced bone mass when compared to age- and gender-matched controls. These findings were apparent also in several subsequent studies, showing reduced BMD and primary osteoporosis in *LRP5* mutation carriers (Lev et al. 2003, Crabbe et al. 2005, Hartikka et al. 2005). However,

our study was the first to describe an extensive and detailed clinical analysis of these individuals and to show that the carriers not only have reduced bone mass but often severe symptomatic osteoporosis that requires treatment.

As missense mutations can be difficult to distinguish from normal polymorphic variation, supporting *in vitro* and *in silico* data is usually needed. The three missense mutations identified in the present study are likely to be disease-causing for a number of reasons. First, these sequence alterations were not present in any of the 478 control samples of Finnish origin. Second, all three nucleotide changes alter amino acid residues that are highly conserved across species. And thirdly, these amino acid alterations are predicted to affect the protein function when using algorithms from web-based SIFT and PolyPhen programs.

The three missense mutations are located in different parts of the LRP5 protein; p.R570W in the second YWTD-propeller, p.R925C in the third EGF-domain, and p.R1036Q in the fourth YWTD-propeller (Figure 5). The role of these domains on LRP5 function is not clearly understood and therefore the exact consequences of these mutations on LRP5 function cannot be predicted. The reported OPPG-associated *LRP5* mutations have been located along the entire gene with no recognizable hot spot areas, suggesting that mutations in any or all domains result in the same phenotype. However, the interesting finding that one of our study subjects in the large Finnish family was compound heterozygote for p.R570W and p.R1036Q but completely lacked the eye phenotype typical for OPPG patients raises the question about the importance of different LPR5 domains on ocular function. A patient with an autosomal recessive FEVR, characterized by ocular abnormalities, has been reported to have the p.R570Q mutation in *LRP5* (Jiao et al. 2004). This mutation is located in the same amino acid residue as the p.R570W mutation found in our study and therefore highlights the importance of this functional domain for the development of the eye phenotype in OPPG and FEVR. On the other hand, the location of the p.R1036Q mutation in the fourth YWTD-propeller suggests that this domain might be crucial for the bone phenotype but not for the eye phenotype. In addition, the Wnt/Frizzled complex binds to the same domain where p.R570W and p.R925C are located suggesting an explanation for the bone phenotype in these patients. In contrast, the role of EGF-domains in LRP5 is poorly understood and the p.R925C mutation located in this domain and the severity of the patient's phenotype, especially as she is a heterozygote mutation carrier, raises the question about the importance of this EGF-domain. Based on predicted 3D LRP5 protein crystal structure, most OPPG-associated *LRP5* mutations occur in the core of the protein and are likely to cause destabilization in protein folding (Toomes et al. 2004). In addition, those mutations located in the surface area of YWTD- and EGF-domains of LRP5 might disrupt functionally important protein-protein interactions (Toomes et al. 2004).

1.3.1. Wnt/ beta-catenin signaling assays (III)

To further characterize the role of p.R570W, p.R925C and p.R1036Q mutations on LRP5 function, we performed Wnt signaling assays to test the effect of these mutations on Wnt signal transduction. This was measured using a luciferase based reporter assay described previously by Ai and co-workers (2005). HEK293T cells were transfected with wild type or mutated LRP5 constructs according to the patient and carrier genotypes: R570W/R570W, R570W/R1036Q, R570W/WT, R925C/WT, and R1036Q/WT. In addition, R925C/R925C and R1036Q/R1036Q genotypes were generated although these were not seen in any of the study subjects.

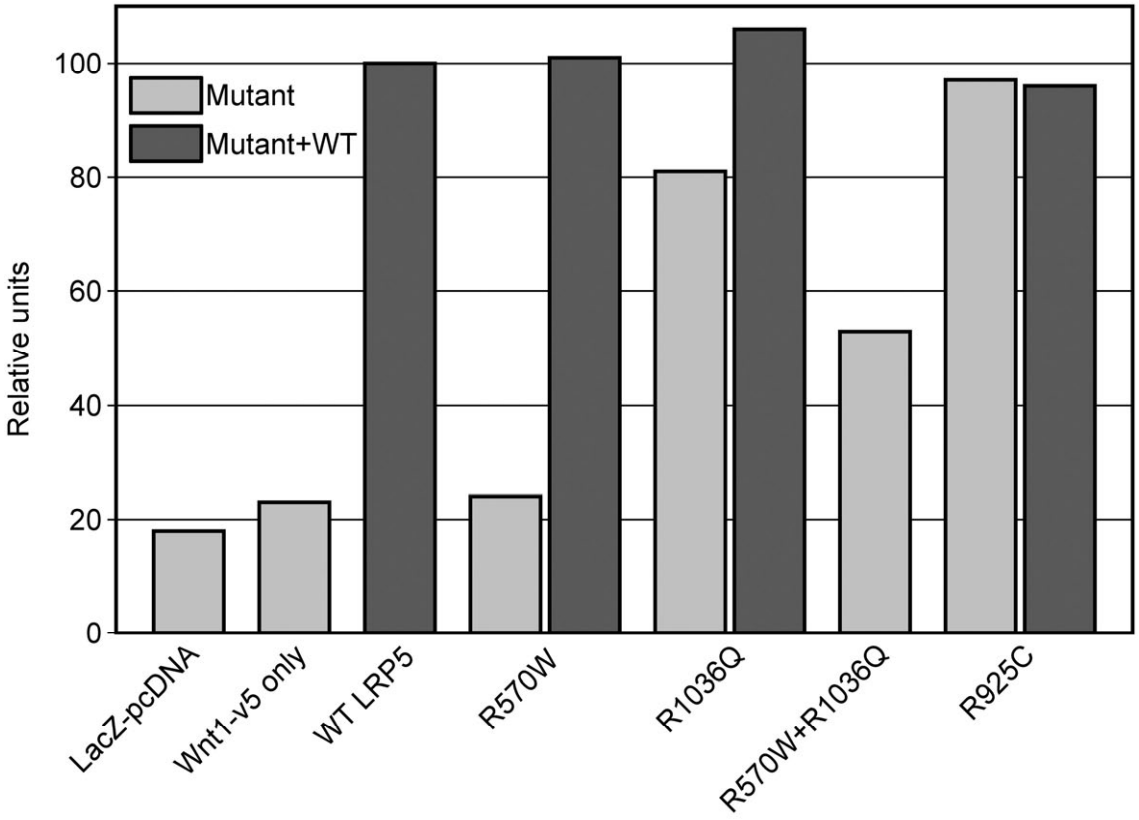

Figure 7. *Luciferase activities from the Wnt/beta-catenin signal transduction assays in HEK293T cells expressing WT or mutant constructs.*

The Wnt/β-catenin signal transduction was significantly reduced only in cells transfected with vector combinations mimicking homozygous and compound heterozygous genotypes for p.R570W and p.R1036Q mutations (Figure 7). Signal transduction was completely lost in the case of homozygous p.R570W, but only slightly reduced in the case of homozygous p.R1036Q. The R570W/R1036Q genotype, as seen in the compound heterozygote patient, resulted in signal transduction intermediate to WT and homozygous p.R570W. Co-transfection of WT and mutant constructs, mimicking a heterozygous state, resulted in signal transduction levels equal to those seen with WT LRP5. Somewhat surprisingly, the p.R925C mutation did not alter the

signal transduction, suggesting an alternative pathological mechanism for this mutation. This also implies that in addition to reduced signal transduction, other pathomechanisms can also affect the skeletal phenotype.

Our results from the Wnt/β-catenin signal transduction assays are in accordance with the results reported in the paper by Ai and co-workers (2005). Notably, the reduced signal transduction capacity of the p.R570W mutation resembles that seen with the FEVR associated p.R570Q mutation, located in the same amino acid residue (Ai et al. 2005).

1.3.2. Immunofluorescent LRP5 trafficking assays (U)

The post-translational modification, trafficking, and localization of OPPG- and HBM-associated *LRP5* mutations have previously been studied using vectors that over-express a truncated form of LRP5 protein lacking the transmembrane and cytoplasmic domains (Ai et al. 2005a and 2005b). In order to study the effect of the identified *LRP5* mutations on the subcellular trafficking and localization of corresponding proteins we performed a series of immunofluorescent assays. Instead of using the truncated forms of LRP5 we used myc-tagged LRP5 constructs expressing a full-length protein and monitored their subcellular localization using an anti-myc antibody.

We performed a series of transfections with WT and mutant constructs in different cell lines and made numerous different immunofluorescence stainings in order to obtain reliable and repeatable results. However, the transfection efficiency of the experiments was unexpectedly low, especially when compared to the GFP control. The few transfected cells over-expressing the WT LRP5 protein showed LRP5 localization in the plasma membrane as expected. Our experiments with the mutated LPR5 constructs, however, resulted in no or weak fluorescent signal. As the number of transfected cells was low, we could not get conclusive evidence for different sub-cellular localization of WT and mutant proteins. In light of previous studies this would have been anticipated, as the truncated mutant LRP5 proteins, although normally post-translationally modified, have been shown to traffic less efficiently to the plasma membrane than the WT LRP5 (Ai ei al. 2005).

Our problems with these experiments might be due to several reasons. Microscopic visualization of the transfected cells showed morphological changes and protein aggregates indicating a level of stress when culturing the cells. It is thus possible that over-expression of the constructs lead to abnormal folding and aggregation of the LRP5 protein and, eventually, to breakdown of the cell. This hypothesis would explain the low transfection efficiency seen in the experiments. Truncated forms of LRP5 should be used in future trafficking assays as has been reported in previous protocols.

1.4. LRP5 and serotonin (III)

Recent studies have revealed an unexpected connection between the bone and the gut, as *LRP5* was suggested to be responsible for the regulation of bone formation through a gut-derived serotonin (Yadav et al. 2008). *LRP5* deficient mice (*Lrp5*$^{-/-}$) were shown to have elevated serum serotonin levels and this was also seen in three OPPG patients with homozygous *LRP5* mutations and in one obligate OPPG mutation carrier (Yadav et al. 2008). Encouraged by these findings, we measured serum serotonin levels from stored blood samples obtained from our Finnish OPPG patients and their family members.

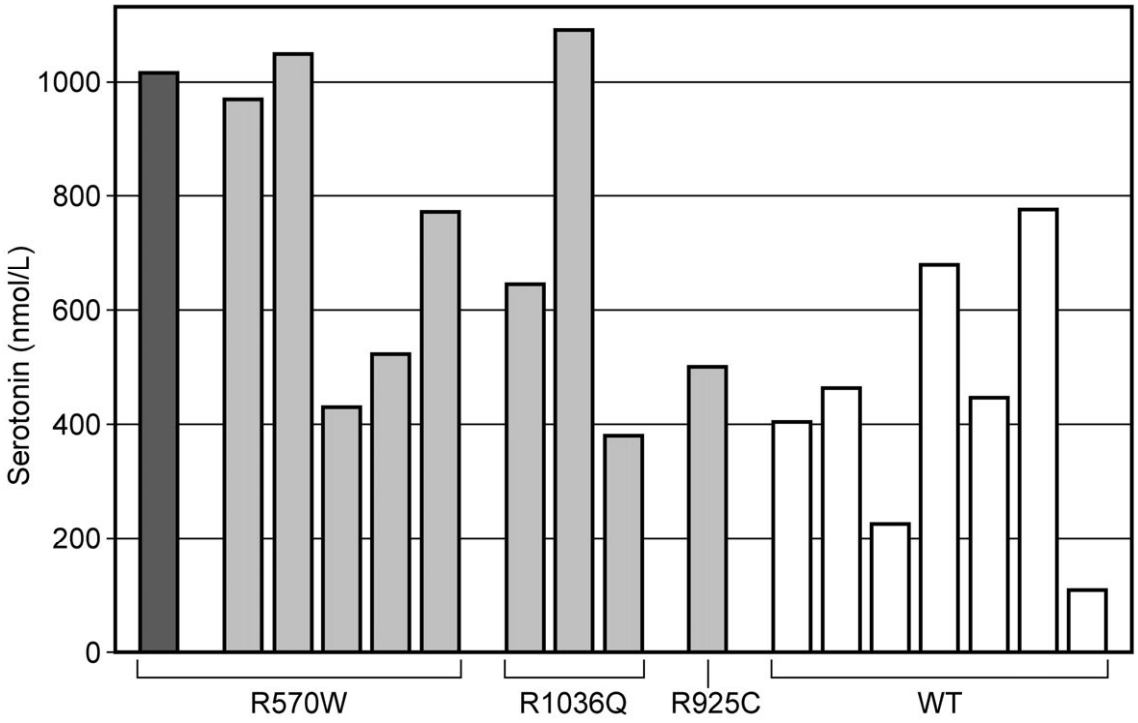

Figure 8. *Serum serotonin concentrations in 17 subjects with and without LRP5 mutations. The light grey bars represent the heterozygote mutation carriers.*

Serum serotonin levels were measured in 17 subjects of whom one was homozygous for the p.R570W mutation, nine were OPPG mutation carriers (heterozygous for p.R570W, p.R1036Q or p.R925C), and seven were mutation negative individuals. There was notable variation in the measured serotonin levels between the samples, but a clear trend towards higher serotonin concentrations in the mutation positive individuals was seen (Figure 8). In the OPPG patient homozygous for the p.R570W mutation the serum serotonin concentration was 1016 nmol/l, whereas the level in mutation negative individuals was 109-776 nmol/l. The OPPG mutation carriers' serum serotonin levels settled between the previous two groups (380-1091 nmol/l) as expected. No clear correlation between the serotonin levels and BMD values of individuals was seen.

The serum serotonin levels differed quite substantially between individuals with the same genotype. The differences might be due to long storage of the blood samples, differences in the uniformity of sample preparations, or normal variability in serotonin concentrations over time. The latter is the most likely explanation as some variability of serotonin levels was also seen in the three OPPG patients described in the paper by Yadav and co-workers (2008).

Mödder and co-workers (2010) performed a population-based study on 275 women and noticed that peripheral serum serotonin levels are inversely associated with numerous BMD values and structural parameters. In our study we could not detect a clear correlation between serum serotonin levels and BMD values, but this was probably due to small sample size and therefore lack of statistical power.

To understand the role of gut-derived serotonin and LRP5 on bone formation, Yadav and co-workers (2008) performed a series of experiments and presented a model of the LRP5-serotonin pathway (Figure 9). Serotonin (5-hydroxytryptamine) is a biogenic amine that functions as a neurotransmitter in the central nervous system and as a hormone in the gastrointestinal tract where 95% of it is produced (Gershon et al. 1990). Because of the blood-brain barrier, these two forms of serotonin are never in contact but instead have different functions in the body. An enzyme called tryptophan hydroxylase 1, encoded by the *TPH1* gene, is responsible for the peripheral serotonin production in the duodenum (Gershon and Tack 2007). Peripheral serotonin binds to, among other proteins, the HTR1B receptor in osteoblasts and activates a signaling cascade which in the end increases the expression of *CyclinD1*, a gene essential for the cell cycle (Yadav et al. 2008). Enhanced *CyclinD1* activation increases the proliferation of osteoblasts and eventually the accrual of bone mass (Yadav et al. 2008).

The results from our study on Finnish OPPG patients and their family members strongly support the findings of Yadav and co-workers (2008) describing the LRP5-serotonin dependent regulation of bone mass. In addition, studies on patients with gain-of-function mutations in *LRP5* causing the high bone mass (HBM) phenotype have revealed reduced plasma serotonin levels (Frost et al. 2010). These findings together give rise to a potential therapeutic pathway for drug development against bone loss. Indeed, studies on *LRP5* deficient (*Lrp5*$^{-/-}$) mice have revealed that gut-specific activation of *Lrp5* or inactivation of *Tph1* increase bone mass and prevent ovariectomy-induced bone loss in mice (Yadav et al. 2008 and 2010). Characterization of the mechanisms involved in this novel LRP5 and serotonin dependent bone formation is still ongoing and contrasting findings have also been reported (Bliziotes 2010). Therefore, further work is required to understand the biochemical pathways and the feedback loops involved in the regulation of LRP5-associated bone formation.

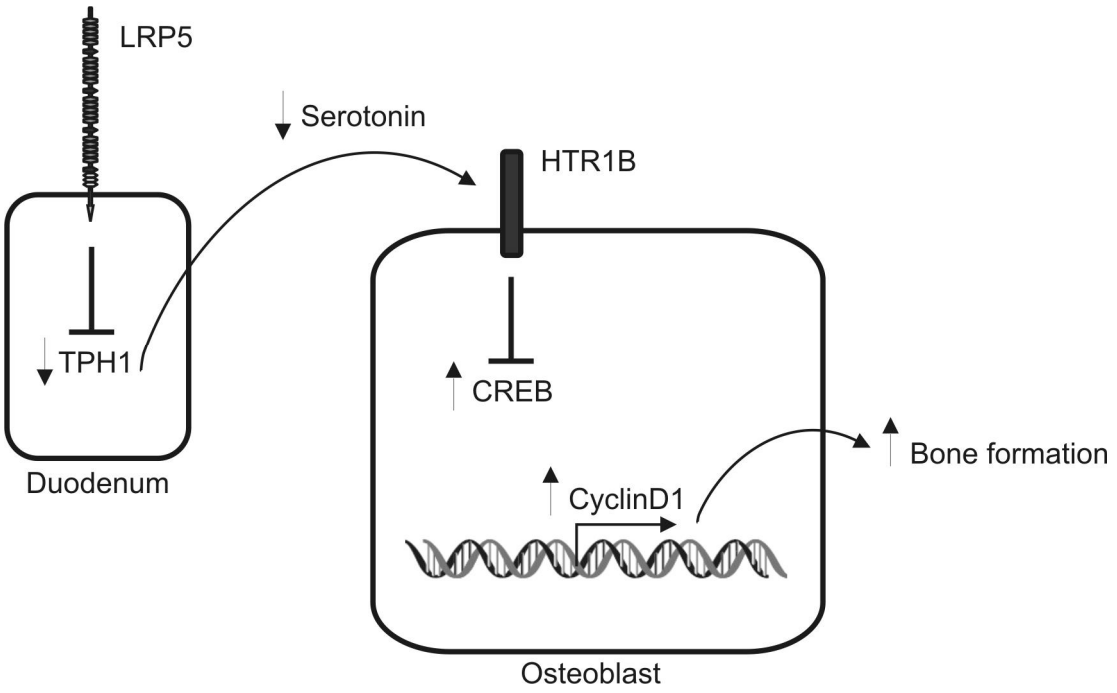

Figure 9. *A schematic model of the LRP5-serotonin dependent regulation of bone mass. LRP5 activation inhibits the expression of TPH1 and regulates the peripheral serotonin levels. A decrease in serotonin levels results in less HTR1B signaling in the osteoblasts, which in turn increases cell proliferation through increased CREB binding and CyclinD1 expression. Adapted from the publication by Yadav and Ducy (2010).*

2. *LRP5* variation and metabolic characteristics

Genes together with environmental factors contribute to the risk of metabolic syndrome, a cluster of risk factors (visceral obesity, insulin resistance, dyslipidaemia, and hypertension) predisposing to type 2 diabetes and cardiovascular disease (Andreassi 2009). Mutations in the genes encoding proteins such as apolipoprotein E (ApoE) and the low-density lipoprotein receptor (LDLR) have been shown to cause hyperlipidaemia and atherosclerosis (Wouters et al. 2005). Studies on LRP5 have long focused solely on bone metabolism, but recent studies have emerged with a new focus towards lipid and glucose metabolisms. Fujino and co-workers (2003) demonstrated the essential role of LRP5 in normal cholesterol metabolism and glucose-induced insulin secretion. They demonstrated that *Lrp5* deficient mice, fed with a high-fat diet, had elevated cholesterol levels due to decreased hepatic clearance of chylomicron remnants. In addition, the significant glucose intolerance seen in these mice was due to decreased insulin secretion but not insulin resistance. They further demonstrated that the cholesterol levels were even higher in mice lacking both *Lrp5* and *ApoE*, suggesting that LRP5 has an important role in the ApoE-dependent catabolism of lipoproteins (Magoori et al. 2003).

Association studies with common polymorphic variations in *LRP5* have further elucidated the role of LRP5 on metabolic risk factors. A study of 1873 Caucasian people from 405 families revealed an association between intronic *LRP5* SNPs and obesity (Guo et al. 2006). Moreover, the p.A1330V variant showed association with high serum total cholesterol levels in Japanese men (Suwazono et al. 2006a). Further studies from the same authors revealed that the combination of this p.A1330V variant with the p.C1429T variant in *GNB3*, a gene coding for the G-protein beta-3 subunit, even increases the risk for hypercholesterolaemia (Suwazono et al. 2007). Another Japanese study of 1444 men and 1161 women with elevated blood pressure revealed an association between the *LRP5* p.Q89R variant and hypertension in females (Suwazono et al. 2006b).

2.1. Metabolic characteristics in the Finnish OPPG family (III)

The evident role of LRP5 in lipid and glucose metabolism led us to assess the Finnish OPPG patients and their family members, described in the previous chapter, for metabolic characteristics. The two OPPG patients and 15 of their family members, together with the one additional patient with a heterozygous *LRP5* mutation, were assessed for medical history, lipid profile, and glucose metabolism. The two OPPG patients were homozygous for the p.R570W mutation, ten were heterozygous carriers of either p.R570W, p.R1036Q, or p.R925C and six had no mutations in *LRP5*.

Glucose tolerance tests showed an abnormal glucose tolerance in seven (7/12; 58%) individuals with an *LRP5* mutation (Table 9). The two patients with OPPG had diabetes. All three individuals heterozygous for p.R1036Q and the one individual heterozygous for p.R925C had impaired glucose tolerance, whereas this was only seen in one of the six p.R570W mutation carriers. Only one of the seven heterozygous individuals was obese (BMI 33.2 kg/m^2), the remaining six were considered normal weight or slightly overweight (BMI 22.2-27.4 kg/m^2). Blood biochemistry measurements indicated that the glucose intolerance in these individuals was not due to insulin resistance but a consequence of an impaired pancreatic beta-cell function. This resembles maturity onset diabetes of the young (MODY), a monogenic type of diabetes caused by mutations in at least six different genes (Vaxillaire and Froguel 2008). In the six individuals without *LRP5* mutation glucose intolerance was seen in two (2/6; 33%). Although the prevalence of abnormal glucose tolerance in individuals with an *LRP5* mutation was notably high, due to the small sample size it did not differ statistically when compared to the individuals without *LRP5* mutation.

The lipid profiles (Table 9) in individuals with *LRP5* mutations resembled those measured in mutation negative subjects. The two patients with OPPG had normal lipid values whereas seven (7/10; 70%) of the heterozygotes had hypercholesterolaemia or

were already on cholesterol-lowering medication. In mutation negative family members the cholesterol levels indicated mild hypercholesterolaemia in five individuals (5/13; 38%), one (1/18; 6%) was already on cholesterol-lowering medication.

Table 9. *Lipid profiles and glucose tolerance in 12 patients with LRP5 mutations.*

Patient no	Genotype	Glucose tolerance	Total cholesterol (mmol/l)	BMI (kg/m^2)	Fat %
#1	R570W / R570W	Diabetes	4.3	22.2	38.7
#2	R570W / R570W	Diabetes	3.9	33.2	35.7
#3	R570W / WT	Normal	3.2	22.0	29.9
#4	R570W / WT	Normal	4.1	21.7	36.0
#5*	R570W / WT	Normal	4.6	27.4	35.6
#6*	R570W / WT	Normal	5.1	27.6	NA
#7	R570W / WT	Diabetes	6.8	26.8	NA
#8	R570W / WT	Normal	3.9	25.1	20.0
#9*	R1036Q / WT	Diabetes	5.6	27.4	27.5
#10	R1036Q / WT	Impaired	6.3	26.3	37.9
#11*	R1036Q / WT	Diabetes	5.0	25.7	37.8
#13*	R925C / WT	Diabetes	3.6	26.7	33.6

BMI, body mass index; NA, data not available, WT, wild type. Reference range for total cholesterol is <5.0 mmol/l. Patients on cholesterol-lowering medication are marked with an asterisk. Fat% was determined by DXA.

Even though the prevalence of glucose intolerance and high cholesterol levels seemed to be elevated in individuals with an *LRP5* mutation, there was no statistical power to detect significant differences. The sample size in our study was relatively small, with only two homozygotes, ten heterozygotes, and six individuals without mutations. Increasing the number of study subjects in the original family might have revealed more *LRP5* mutation carriers, and clinical assessment of additional subjects could have given sufficient power to confirm the differences seen in metabolic parameters.

However, our results, showing a trend towards glucose intolerance and higher cholesterol levels in *LRP5* mutation carriers, are in line with the previous findings from *Lrp5* deficient mice (Fujino et al. 2003, Magoori et al. 2003). The prevalence of these metabolic findings in *LRP5* mutation carriers needs to be studied further in larger cohorts and in different populations. As the etiology underlying aberrant glucose and cholesterol metabolism on the population level is most probably multigenic, future studies are needed to explore novel pathways and interactions between *LRP5* and other genes. Interestingly, a recent study by Mani and co-workers (2007) revealed a connection between LRP6 and several metabolic risk factors. A missense mutation

(p.R611C), located in a highly conserved residue of the second EGF domain of LRP6, was found in a family with autosomal dominant early onset coronary artery disease, features of metabolic syndrome, and osteoporosis. The same year, findings from another study revealed an association between a common polymorphic *LRP6* variant (p.I1062V) and late-onset Alzheimer's disease (De Ferrari et al. 2007). As the interplay between *LRP5* and *LRP6* is already evident in bone metabolism (Holmen et al. 2004), the interaction between these genes in the pathways of extra-skeletal metabolisms should be studied further.

Circulating gut-derived serotonin levels were elevated in the family members with an *LRP5* mutation (Figure 8). The role of brain-derived serotonin has been demonstrated in the regulation of bone mass and appetite (Yadav et al. 2009) but the possible role of this "other" serotonin in the regulation of lipid and glucose metabolism still remains unclear. Intriguingly, recent studies revealed an unexpected finding showing that the skeleton works as an endocrine organ by regulating blood glucose levels through osteocalcin (Ferron et al. 2010). They showed that osteocalcin, expressed solely in the osteoblasts, regulates insulin production by pancreatic beta-cells and, on the other hand, insulin sensitivity through adiponectin release from adipocytes. Further studies are still needed to evaluate the role of serotonin and osteocalcin in lipid and glucose metabolism.

2.2. The role of LRP5 in adrenal cortex function (II)

Adrenal glands are essential hormone secreting glands located immediately anterior to the kidneys. The adrenal gland consists of two separate parts, the medulla and the cortex, which have their own distinct endocrine functions. Adrenal cortex produces, among many other hormones, androgens such as dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS), which are required for adrenarche and development of the typical physiological changes seen in puberty (Auchus and Rainey 2004). Adrenarche is regarded as premature if the typical signs (growth of pubic or axillary hair, oily hair and skin, acne, adult-type sweating and body odor) occur before the age of 8 years in girls or before 9 years in boys and if the signs are accompanied by elevated DHEA/DHEAS levels (Ibáñez et al. 2000a). Patients with PA have an increased risk of metabolic syndrome and cardiovascular diseases (Ibáñez et al. 2009). In addition, children with PA have elevated BMD levels compared to age-matched pre-pubertal controls (Ibáñez et al. 2000b, Utriainen et al. 2009).

Wnt signaling is present in almost every tissue and it plays a role also in the adrenal cortex cells (Suwa et al. 2003). High levels of LRP5 expression have been detected in the adrenal cortex, suggesting a pivotal role in cortical function (Kim et al. 1998). As mentioned previously, the role of common polymorphic *LRP5* variants in the risk

factors for metabolic syndrome was shown recently (Guo et al. 2006, Suwazono et al. 2006a and 2006b). Moreover, a highly conserved missense mutation in *LRP6* was found in a family with early-onset coronary artery disease and features of metabolic syndrome (Mani et al. 2007). These genetic findings, together with the preliminary results from our own studies (Study III) and clinical metabolic findings in children with PA, encouraged us to study the role of LRP5 in the pathogenesis of PA.

We sequenced the exons and flanking intronic and UTR regions of *LRP5* in DNA samples from 73 children with PA and 97 healthy pre-pubertal controls. No apparent disease-causing mutations were found in either group. However, 19 polymorphic sequence variants were identified and those with an allele frequency >5% were selected for association analyses. The p.A1330V variant was the only missense change, whereas the remaining four variants were synonymous (p.F549F, p.E644E, p.N740N, and p.V1119V).

No significant differences in the distribution of the 19 variants were noticed between the two groups. Association analyses with the five common *LRP5* variants revealed several significant associations in both groups. In children with PA (Table 10) the p.F549F variant was significantly associated with low baseline cortisol levels, whereas the p.N740N and p.V1119V variants showed association with higher HDL cholesterol levels and higher systolic blood pressure, respectively. No association was seen between the genotypes in adrenocortical hormone profile or metabolic characteristics of these children.

Table 10. *LRP5 variants associated with clinical characteristics in PA subjects.*

Variant	Characteristic	Major allele	Minor allele	*P*
p.F549F	Cortisol (nmol/l)	249 (224-278)	149 (104-214)	0.002
p.N740N	HDL cholesterol (mmol/l)	1.4 (1.3-1.4)	1.6 (1.3-1.9)	0.03
p.V1119V	Systolic BP (mmHg)	103 (100-105)	108 (104-112)	0.02

BP, blood pressure; HDL, high-density lipoprotein.

Interestingly, association with several metabolic characteristics was seen in the control group (Table 11). A significant association was seen between the p.A1330V variant and the total/HDL cholesterol ratio. The p.A1330V variant was also associated with total cholesterol, LDL cholesterol, and DHEAS levels. A slight association was also seen between p.A1330V and higher weight-for-height ratios. The p.V1119V variant showed association with higher total cholesterol and LDL cholesterol levels. Control subjects with the p.F549F variant had a tendency towards higher systolic blood pressure.

We presented the first study describing unfavourable metabolic characteristics in children with common *LRP5* variants. Notably, the effect of these variants is apparent already in early childhood highlighting the important role of LRP5 in metabolic

features. Our findings are in concordance with those seen in other studies (Fujino et al. 2003, Suwazono et al. 2006a) and in our own studies with OPPG patients and *LRP5* mutation carriers (Study III). However, one finding from the present study differs from the previous findings by Suwazono and co-workers (2006c). They showed that the p.A1330V variant associates with low blood pressure in the Japanese population. Our own results, however, showed higher blood pressure levels in subjects with the p.F549F and p.V1119V variants. The contradictory results might be due to differences in age or ethnicity between study cohorts. Also, it is possible that due to the relatively small sample size used in our study we did not have enough statistical power to reach significant associations.

Table 11. *LRP5 variants associated with clinical characteristics in control subjects.*

Variant	Characteristic	Major allele	Minor allele	*P*
p.F549F	Systolic BP (mmHg)	100 (98-102)	108 (95-121)	0.04
p.V1119V	Cholesterol (mmol/l)	4.1 (4.0-4.3)	4.5 (4.2-4.8)	0.04
	LDL cholesterol (mmol/l)	2.4 (2.3-2.5)	2.7 (2.4-3.0)	0.03
p.A1330V	Cholesterol (mmol/l)	4.2 (4.0-4.3)	4.7 (4.1-5.4)	0.02
	LDL cholesterol (mmol/l)	2.4 (2.3-2.5)	2.9 (2.3-3.6)	0.02
	Total/HDL cholesterol (mmol/l)	2.9 (2.8-3.0)	3.6 (2.5-4.7)	0.007
	DHEAS (µmol/l)	0.8 (0.7-0.9)	1.4 (0.8-2.3)	0.01
	Weight-for-height (%)	106 (103-109)	112 (103-121)	0.09

BP, blood pressure; DHEAS, dehydroepiandrosterone; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

The similarities in genotypes between the children with PA and in the control group clearly indicate that *LRP5* does not contribute to the pathogenesis of PA. However, the lower cortisol levels seen in PA subjects with the p.F549F variant and the elevated DHEAS levels seen in control subjects with the p.A1330V variant indicate that LRP5 function may modulate the adrenal cortex hormone synthesis through Wnt or other signaling pathways.

To conclude, we demonstrated that common polymorphic variants in *LRP5* associate with unfavorable metabolic characteristics. Changes in the lipid profile are already apparent in pre-pubertal children. Further studies with larger study cohorts are needed to study the role of *LRP5* variants on cholesterol and blood pressure levels and in the development of metabolic syndrome.

CONCLUSIONS AND FUTURE PROSPECTS

This thesis describes several novel findings concerning the role of LRP5 in bone mass development and in glucose and lipid metabolism.

We demonstrated that LRP5 has a major role on bone accrual and in peak bone mass development. Our studies showed that homozygous mutations in *LRP5* cause severe osteoporosis and even in a heterozygous state increase the risk for osteoporosis. Obligate *LRP5* mutation carriers have significantly reduced BMD and a higher number of spinal compression fractures compared to individuals without mutations. Furthermore, we demonstrated that the *LRP5* mutation positive individuals have a clear trend towards higher circulating serotonin concentrations, supporting the model for LRP5-serotonin dependent regulation of bone mass. In addition, we were the first group to demonstrate that a common polymorphic variant in *LRP5* contributes to peak bone mass development, an important determinant of bone mineral density in later life. The role of rare *LRP5* variants in bone metabolism and in the ethiology of fractures remained unclear.

In addition to the evident role of LRP5 in bone metabolism, we demonstrated that LRP5 has also a distinct role in glucose and lipid metabolism. Our studies revealed an unexpectedly high prevalence of glucose intolerance and dyslipidaemia in subjects with *LRP5* mutations. In addition, we demonstrated that common polymorphic *LRP5* variants associate with unfavorable metabolic characteristics such as high cholesterol levels and blood pressure in healthy pre-pubertal children, indicating that the effect of these variants is evident already in childhood. Our concurrent studies showed that *LRP5* variants do not contribute to the pathogenesis of premature adrenarche.

Bones have typically been thought of as calcified structures with a sole function as the backbone for the body and producer of blood cells. However, this thought has now changed permanently as new evidence has revealed an additional role for bone in lipid and glucose metabolism. Moreover, the surprising observation that gut-derived serotonin regulates bone formation makes the function of bone and the regulation of bone formation even more intriguing.

The prevailing hypothesis suggests that a combination of multiple rare variants predispose to common traits. Therefore, a candidate gene based analysis would be needed to identify the combination of variants predisposing to fractures, and ultimately, osteoporosis. Further studies will be needed to evaluate the role of LRP5 and impaired Wnt signaling in the development of osteoporosis, fractures, and aberrant glucose and lipid metabolism. It is important to recognize the unknown

number of *LRP5* mutation carriers as they have not only low BMD but also an increased risk for metabolic syndrome. Therefore, it is important to characterize these variants and to determine their functional metabolic consequences. Finally, the outcome of future research might help in the development of treatments for bone disorders and also against those extra-skeletal implications caused by altered Wnt signaling.

ACKNOWLEDGEMENTS

This study was carried out at the Folkhälsan Institute of Genetics, the Department of Medical Genetics, University of Helsinki, and the Hospital for Children and Adolescents, Helsinki University Central Hospital, during the years 2005-2011. I wish to express my sincere gratitude to all the patients and families for participating in these studies and making this thesis possible. I am grateful to all the people who contributed to this project, especially:

Professors Per-Henrik Groop, Anna-Elina Lehesjoki, and Päivi Peltomäki, and Docent Irma Järvelä, the former and current heads of the Department of Medical Genetics and the Folkhälsan Institute of Genetics, for providing excellent research facilities and working environment in Biomedicum Helsinki.

The Academy of Finland, the Finnish Medical Foundation, the Folkhälsan Research Foundation, Helsinki University Hospital research funds, the Sigrid Jusélius Foundation, Ministry of Education, the Foundation for Paediatric Research, the Päivikki and Sakari Sohlberg Foundation, the Emil Aaltonen foundation, the Finnish Concordia fund, the Finnish Society of Medical Genetics, Helsinki University Chancellor's travel grants, the Oskar Öflund Foundation, and the Tervakoski Foundation for Student Grants, for financially supporting this project and giving me the opportunity to attend numerous international conferences.

Docent Outi Mäkitie, my supervisor and the head of our amazing research group, is gratefully appreciated for giving me the opportunity to join her research group and introducing me to the fascinating world of bones. Your support and unbelievable ability to handle "the package", supervising numerous PhD students and still having time for your work as a clinician, has carried me through this thesis project. Your enthusiastic approach to research has taught a great deal to all of us who have had the pleasure to work in your group.

Professor Anna-Elina Lehesjoki, my co-supervisor and the head of Folkhälsan Institute of Genetics, is sincerely thanked for her never-ending support. I have been privileged to work in your group in numerous research projects during these almost ten years. You have been more than just a "boss" for me as, even with your busy schedule, you have always had time to be there for me and be supportive during the ups and downs of my personal life.

Professor Heikki Kröger and docent Sirpa Kivirikko, the official reviewers, are thanked for their valuable comments and ideas on how to improve the thesis.

Jodie Painter, the former colleague from Down Under, for revising the language of this thesis and for your support in the last moments of writing and re-writing of this thesis. I also want to thank you for all those friendly and encouraging conversations during FIG times. Pekka Paavola is also acknowledged for re-drawing the luciferase and serotonin graphs of this thesis.

Co-authors Kirsi Auro, William G. Cole, Tero Kivelä, Mairi Kuris, Jarmo Jääskeläinen, Saila Laakso (née Lappalainen), Ulla Lahtinen, Christine Laine, Eliisa Löyttyniemi, Mervi Mäyränpää, Tero Saukkonen, Mirja Somer, Sanna Toiviainen-Salo, Pauliina Utriainen, Piia Uusen, Raimo Voutilainen, Matti J. Välimäki, and Ville-Valtteri Välimäki, for their valuable contributions to this thesis work. Ulla is also thanked for helping me with the cell work and for all those profound discussions about the cat's mysterious life. Mirja is especially acknowledged for sharing some historic details during our conversation on the bathroom floor at ESHG Amsterdam 2006.

Dr. Matthew Warman, our colleague from Boston, Massachusetts, USA, for providing us with expression vectors for the Wnt signalling assays and giving me the opportunity to visit his lab in Case Western Reserve University, Cleveland, Ohio, USA. His former student Minrong Ai from CWRU, is especially acknowledged for teaching me the secrets behind Wnt signalling assays and making my trip to Cleveland a joyful experience.

The "Skele Girls" Päivi Kilpinen-Loisa, Saila Laakso (née Lappalainen), Christine Laine, Kati Markula-Patjas, Mervi Mäyränpää, Sanna Toiviainen-Salo, and Helena Valta, for the joyful moments together. I especially enjoyed the do-it-yourself cooking experience in "Lotta's". Your support before my presentations in Montreal was highly appreciated. Mervi is especially thanked for her friendship and support during the "baby-project". I have enjoyed all our conversations during the numerous coffee breaks and I really appreciate you being there when I needed a shoulder to cry on. It's a priviledge to be your friend.

Mira Aronen, Mia Gagneur, Jenny Kivinen, Mairi Kuris, Ahmed Mohamed, Riikka Mäkitie, and Piia Uusen are thanked for their excellent technical assistance in the lab. Mairi is especially thanked for her contribution at the beginning of this project and being my right (and left) hand in the lab. You really kept ME busy! Your cheerful attitude and organizational skills made my part of the work easy. I'm glad that our friendship has continued after your FIG times.

Nea Boman, Sinikka Lindh, and Päivikki Rissanen, are appreciated for their help with contacting patients and families, collecting blood samples, and keeping the files

updated. Sinikka is especially thanked for the long conversations and help in the drawing of pedigrees.

Solveig Halonen, Stephan Keskinen, Aila Riikonen, Marjatta Valkama, and Jaana Welin-Haapamäki, our secretaries and office staff, are thanked for kindly helping me with so many practical matters during the years. Solveig, thank you for your unique positivity towards life!

All the present and former friends at the Folkhälsan Reseach Institute are warmly thanked for making these almost ten years very enjoyable. You really made every morning worth coming to work. There are so many people I would like to thank, but especially the following are warmly thanked: my dear PhD fellows Milja Kaare, Mervi Kuronen, Vilma-Lotta Lehtokari, and Saara Tegelberg for their support, mind-blowing coffee table conversations and friendship. Ann-Liz Träskelin (Grattis! Bra jobbat!) is warmly thanked for her encouraging words, guidance and hugs, and for always being there for me. Nina Aula is greatly thanked for her contagious joyful spirit and for reminding me that there's also life beyond science. Mervi, ALT and Naula, our Härkälinna organizing committee, are also thanked for the memorable and hilarious moments when planning and executing the trip. I would also like to acknowledge the following people: Nina Ekman (née Halla) for sharing the pipeting sessions during Cohen; Reetta Jalkanen for her cheerful attitude and help in the many dissertation-related questions; Kaisa Kettunen for baby talk; Maria Kousi for encouraging words; Inken Körber and Anni Laari for daily discussions; Maria Lunkka-Hytönen for help with MLPA; Otto Manninen for wine&dine tips (no ethnic food today!); Laura Mustaniemi (née Waris) for the unforgettable laughs (I really miss it); Anna Naukkarinen for sharing the stressful last moments; Maria Sandbacka for everyday discussions and whole-hearted support; Jaakko Sarparanta for having an answer to all those universal questions conserning computers, methods, birds etc.; and Hanna Västinsalo for the opportunity to participate in The Movie and for her cheerful attitude torwards research. Our experienced and highly valued senior scientists; Peter Hackman for cheerful "Happy" Monday –moments; Tarja Joensuu for all the support and mentoring; Outi Kopra for the help with transfections and microscopy; and Anne Polvi for lunch break discussions. I also want to acknowledge the former PhD students Kirsi Alakurtti, Anna-Kaisa Anttonen, Nina Aula, Kati Donner (I miss your singing), Riikka Hämäläinen, Juha Isosomppi (my chat buddy), Jukka Kallijärvi, Juha Kolehmainen, Liina Nevalaita (née Lonka), Sanna Partanen, Tarja Salonen, Eija Siintola, and Jouni Vesa. Kirsi and Eija are also warmly thanked for their inspiring company and good friendship. A big hug for you all!

Paula Hakala and Teija Toivonen are greatly acknowledged for their ever-helping hand in the lab and patience for answering my questions again and again. Also Hanne Ahola,

Hanna Hellgren, Mia Manu-Niskanen, Merja Soininen, and Marilotta Turunen are thanked for their help in the lab.

Per-Henrik Groop, the former head of our Institute, is greatly appreciated for his enthusiastic personality. I have enjoyed all our corridor conversations. Maija Wessman is thanked for her support and wise words. Maikki Parkkinen is thanked for help with Taqman assays. Anja Koskela, Adile Samaletdin, and Tuula Soppela are greatly thanked for their cheerful spirit and all those numerous coffee table conversations. Minna Pöyhönen is thanked for her company in numerous conferences all around the world.

A huge thanks to all my not work-related friends for the excellent times spent in Tervakoski, Lahti, Turku, and Espoo.

The love and support provided by my family and soon to be (?) relatives cannot be thanked enough. The Juvonen clan (Tuila, Pekko & Anissa, Pertti, Margit, Leeppi, Maukki & Tuula, and Mikko & Sari) are warmly thanked for welcoming me to their family with open arms. Tuila, my “sister-in-law”, is greatly thanked for giving a helping hand when taking care of Neela and always being there for us. Your company during the long flights and in pre- and post-conference sightseeing has brought so many unforgettable memories!

My parents Pirjo and Jorma are warmly thanked for their endless support and love and for the financial support throughout my studies. You provided a loving home, which was the perfect base for my adult life. My brother Juha-Matti and his son Ville are warmly thanked for their support and for being an important part of my life.

Finally, I want to thank my soulmate Tuukka for his love and support and for taking care of our household especially during the long hours of the writing process. You have brought so much joy to my life and especially given me the best present ever, our daughter Neela. Neela is warmly thanked for her never-ending happy spirit, non-stop laughs, hugs and wet kisses. Your unconditional love and company means the world to me.

Espoo, January 2011

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