

# GENETIC FACTORS IN BONE DISORDERS

Osteogenesis imperfecta, juvenile osteoporosis and stress fractures

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## **Hartikka, Heini, Genetic factors in bone disorders. Osteogenesis imperfecta, juvenile osteoporosis and stress fractures**

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### ***Abstract***

Genetic factors and their resulting phenotypes were evaluated in three different bone disorders: osteogenesis imperfecta (OI), juvenile idiopathic osteoporosis (JIO), and stress fractures.

The spectrum of the OI phenotypes caused by mutations in the *COL1A1* and *COL1A2* genes is well defined, but the mechanisms by which the variations affect the hearing phenotype are not well-known. A total of 54 Finnish OI patients with previously diagnosed hearing loss, or aged 35 or more years, were analyzed here for mutations in *COL1A1*, or *COL1A2*. Altogether, 49 mutations were identified, of which 41 were novel. No correlation was observed between the mutated gene, or the mutation type, and the hearing pattern. This indicates that the basis of hearing loss in OI is complex, and is a result of multifactorial, still unknown genetic effects, or of variable expressions of the *COL1A1* and *COL1A2* genes.

JIO presents peri-pubertally as an acute symptomatic osteoporosis (bone pain and fractures) in otherwise healthy children, and no underlying cause has yet been identified for this disorder. Here, the analysis of the low-density lipoprotein receptor-related protein 5 gene (*LRP5*) in 20 patients with JIO revealed two missense mutations (A29T and R1036Q) and one frameshift mutation (C913fs) in 3 of the patients. The *LRP5* gene has recently been shown to be also involved in osteoporosis-pseudoglioma syndrome and a high-bone-mass phenotype.

Stress fractures are a significant problem among athletes and soldiers. Genetic factors may increase the fracture risk, but no susceptibility genes have yet been identified. Seven genes involved in bone metabolism, or pathology, were studied in terms of their roles in stress fracture. No disease-causing, or predisposing variations were found in the candidate gene, or association analyses, but a highly significant association was found between the phenotype and a vitamin D receptor (*VDR*) haplotype, TGT, which is composed of three polymorphic sites, *FokI*, *BsmI* and *TaqI*. We showed that femoral neck stress fractures are associated with a certain *VDR* haplotype, accounting for a five-fold increase in the risk of developing stress fractures, with an associated attributable risk of 12%.

The results of this study show that genetic factors play a role in different pathological bone phenotypes. These findings provide new information on the pathogenesis of the disorders and for the development of genetic testing and targeted treatment for the disorders.

**Keywords:** bone, juvenile idiopathic osteoporosis, osteogenesis imperfecta, stress fractures



*To Jarno and my family*



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Oulu, May 2005

Heini Hartikka

## Abbreviations

A	Adenosine
ALP	Alkaline phosphatase
BMD	Bone mineral density
bp	Base-pair
C	Cytidine
C-	Carboxy
cDNA	Complementary deoxyribonucleic acid
CSGE	Conformation-sensitive gel electrophoresis
dB	Decibel
Dkk	Dickkopf
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FEO	Familial expansile osteolysis
Fz	Frizzled proteins
G	Guanidine
Gly	Glycine
HBM	High bone mass
HL	Hearing level
Hz	Hertz
JIO	Juvenile idiopathic osteoporosis
kb	Kilobase
LDLR	Low-density lipoprotein receptor
LRP5	Low-density lipoprotein receptor-related protein 5
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
N	Amino
NMD	Nonsense-mediated mRNA decay
nt	Nucleotide
OH	Hydroxyl
OI	Osteogenesis imperfecta
OPPG	Osteoporosis-pseudoglioma syndrome

OPG	Osteoprotegerin
PCR	Polymerase chain-reaction
PDB	Paget's disease of bone
PHPV	Persistent hyperplasia of primary vitreous
RANK	Receptor activator for NF- $\kappa$ B
RANKL	Receptor activator for NF- $\kappa$ B ligand
RT-PCR	Reverse transcription PCR
SD	Standard deviation
SDE	Serine-aspartic acid-glutamic acid
SNP	Single nucleotide polymorphism
T	Thymidine
T-score	Standard deviation unit
VDR	Vitamin D receptor
VDRR	Vitamin D-resistant rickets
Wnt	Wingless
X	Any amino acid
Y	Any amino acid
YWTD	Tyrosine-tryptophan-threonine-aspartic acid
Z-score	Standard deviation score

## List of original articles

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

- I Hartikka H\*, Kuurila K\*, K rkk  J, Kaitila I, Gr nman R, Pynn nen S, Hyland JC & Ala-Kokko L (2004) Lack of correlation between the type of *COL1A1* or *COL1A2* mutation and hearing loss in osteogenesis imperfecta patients. *Hum Mutat* 24: 147-154. \*Equal contribution.
- II Hartikka H, M kitie O, M nnikk  M, Doria AS, Daneman A, Cole WG, Ala-Kokko L & Sochett EB (2005) Heterozygous Mutations in the LDL Receptor-Related Protein 5 (*LRP5*) Gene Are Associated with Primary Osteoporosis in Children. *J Bone Miner Res* 20: 783-789.
- III Hartikka H, Pihlajam ki H, Ruohola J-P, Sahi T, Barral S, Ott J & Ala-Kokko L (2005) Vitamin D receptor gene haplotype predisposes to femoral neck stress fractures. Submitted.



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# 1 Introduction

Bone is a highly specialized form of connective tissue. It is a living tissue composed of an organic matrix strengthened by the deposit of calcium salts and bone cells. The organic matrix of bone consists of approximately 90 % type I collagen and 10 % proteoglycans and non-collagenous proteins. There are two forms of bone, termed cortical bone and cancellous bone. The two bone types differ by their primary functions: cortical bone provides mechanical and protective functions, while cancellous bone provides metabolic functions.

Osteogenesis imperfecta (OI) is a heritable disorder characterized by varying degrees of bone fragility, and is related to defects in several other tissues rich in type I collagen. A large number of mutations in the *COL1A1* and *COL1A2* genes, which encode the pro $\alpha$ 1 and pro $\alpha$ 2 chains of type I collagen, have already been characterized. These mutations include single-base mutations causing glycine substitutions, splicing mutations and null allele mutations. In general, mutations that reduce the amount collagen I lead to the mild variant of the disease, OI type I. Mutations that alter the structure of the pro $\alpha$  chains and result in the formation of abnormal molecules, typically lead to more severe phenotypes. Genotype-phenotype correlation studies of OI have mainly focused on the severity of the disease, while correlations between the mutations and a spectrum of phenotypes have not been widely studied.

Secondary osteoporosis is being increasingly recognized in children. Primary osteoporosis is less common and is usually diagnosed as either OI, or juvenile idiopathic osteoporosis (JIO). The gene encoding the low-density lipoprotein receptor-related protein 5 (LRP5) has recently been shown to affect bone mass accrual during growth. LRP5 is expressed in a wide variety of tissues and cells, including osteoblasts, and the protein is involved in the Wnt signalling pathway, which alters bone mass through a primary effect on bone formation. Homozygous inactivating mutations in the *LRP5* gene cause autosomal recessive osteoporosis-pseudoglioma syndrome (OPPG), characterized by severe juvenile-onset osteoporosis and congenital, or early-onset blindness. The *LRP5* gene has also been shown to impact bone mass in mice. Taken together, these data suggest that the *LRP5* gene affects bone mass and may thus play a role in the development of osteoporosis.

Stress fractures represent one of the most common and potentially serious over-use injuries. They are commonly seen in athletes and in military recruits during basic

training. It has been suggested that a genetic component contributes to the predisposition to stress fracture. Furthermore, studies on twins and their family members have established that differences in certain traits, such as bone size and shape, and bone mineral density (BMD), between individuals of the same age are largely attributable to differences in their genes, and not to environmental effects. Sequence variations in the genes that may predispose to stress fractures are involved in bone formation and bone remodelling. However, the genetic defects leading to stress fracture predisposition are not currently known.

Knowledge of the genetic defects behind different bone disorders is important for understanding the pathogenesis of the disorders, for developing genetic testing, and for the development of specific, targeted treatments for these diseases.

The aim of this thesis is to increase the knowledge-base concerning the different genetic factors implicated in disorders affecting bone tissue. The research identifies 49 mutations in the *COL1A1* and *COL1A2* genes causing OI, and their genotypic and phenotypic comparison. In addition, it describes three mutations in the *LRP5* gene causing JIO, and an association between a vitamin D receptor gene haplotype and femoral neck stress fractures.

## **2 Review of the literature**

### **2.1 Bone**

Bone is a rigid, dynamic organ that is continuously shaped and repaired. Bone microstructure is patterned to provide maximal strength with minimal mass. The bone is needed for movement and speed, with properties that meet the contradictory needs of strength and lightness, stiffness and flexibility. Stiffening the rope-like triple-helical fibrils of type I collagen with mineral crystal introduces resistance to bending, and excessive stiffness produces glass-like brittleness. Strength and lightness are achieved by a geometrical structure. This is observed in long bones, where tubular structures have a marrow cavity, and the cortical mass is placed distant from the central long axis, ensuring better resistance to bending. (Seeman 2002).

Bone tissue, the major constituent of the human skeleton, has structural, protective and metabolic functions. The structural functions provide support and insertion sites for ligaments, tendons and muscles. The protective functions, ensured by the skull and thoracic cage, for example, provide physical protection for the brain, and the thoracic and upper abdominal organs. The metabolic functions of bone provide a reservoir of essential minerals and the support of haematopoiesis. (Marks & Hermey 1996, Hughes 2000).

Morphologically, there are two forms of bone, termed cortical (compact) and trabecular (cancellous) bone. Cortical bone is the dense bone that has a predominantly structural, load-bearing function and predominates in the long bones of the skeleton. It is characterized by layers of bone matrix (lamellae) arranged concentrically in cylindrical structures called Haversian systems. Each column surrounds a Haversian canal containing blood and lymph vessels and nerves. Trabecular bone is found inside the cortex and consists of a network of interconnecting plates with perforations through which blood vessels pass. Bone marrow is situated between these plates. Trabecular bone is mainly responsible for the metabolic function of bone. For this reason, it is far more prone to diseases, such as osteoporosis resulting from increased bone remodelling, than cortical bone. (Marks & Hermey 1996, Hughes 2000, Seeman 2002).

### ***2.1.1 Components of bone matrix***

The three major components of bone are bone cells, organic matrix and mineral. The osteogenic cells include osteoblasts, osteocytes and osteoclasts, while the matrix consists predominantly of collagen and proteoglycans. The matrix accounts for approximately one-third of the bone mass. The mineral, accounting for approximately two-thirds of the bone dry weight, is composed of calcium phosphate crystals deposited as hydroxyapatite.

#### ***2.1.1.1 Bone cells***

Three different cell types can be found within bone: the matrix-producing osteoblasts, the tissue-resorbing osteoclasts, and the osteocytes, which represent 90 % of all cells of the adult skeleton.

Osteoblasts are derived from mesenchymal stem cells of the bone marrow stroma. They possess a single nucleus and a morphology that varies from flat to plump, reflecting their level of cellular activity. Osteoblasts form a cell layer over bone surfaces upon which matrix is being formed; osteoblasts produce and secrete the major part of the organic bone matrix and regulate its mineralization. They synthesize type I collagen, which comprises 90-95% of the organic matrix, as well as non-collagenous proteins of the bone matrix, such as proteoglycans, glycoproteins and  $\gamma$ -carboxy glutamic acid-containing (gla) proteins. They also regulate the differentiation and activity of the bone-resorbing osteoclasts. Following a period of secretory activity, osteoblasts either undergo apoptosis, or become embedded in the bone matrix to differentiate into the mature bone cells, known as osteocytes. (Mackie 2003).

Osteocytes can be viewed as highly specialized and differentiated osteoblasts, while osteoblasts have been described as sophisticated fibroblasts (Ducy *et al.* 2000). Osteocytes form an interconnecting network throughout the bone matrix and are in contact with cells located on the bone surface via a network of dendritic processes passing through canaliculi in the bone matrix. Osteocytes are the most abundant cells in bone tissue, but they are smaller than osteoblasts and have lost many of their cytoplasmic organelles. The functions of osteocytes are poorly understood, but there is evidence to suggest that osteocytes sense mechanical stress on the bone and can thus contribute to the bone remodelling process. (Burger & Klein-Nulend 1999).

Osteoclasts, derived from the monocyte/macrophage haematopoietic lineage, develop and adhere to the bone matrix. Like macrophages, osteoclasts are highly migratory, multinucleated, polarized cells, which carry an arsenal of lysosomal enzymes. The mature osteoclast is activated by different signals, leading to the initiation of bone remodelling. The main functional feature of osteoclasts is their ability to dissolve both mineral and the organic matrix. Increased remodelling and osteoclastic activity characterize several metabolic bone diseases, such as osteoporosis, hyperparathyroidism and Paget's disease. (Sommerfeldt & Rubin 2001).

### 2.1.1.2 Collagens

The organic matrix of bone is primarily composed of collagen. Types I, II, III, V and XI collagens belong to the group of fibril-forming collagens. The characteristic feature of fibrillar collagens is that they consist of a long, continuous triple helix, which self-assembles into highly organized fibrils. Type I collagen is the most abundant extracellular protein in bone, representing 90 % of the organic bone matrix. It is also a major constituent of tendons, ligaments, skin, teeth and fasciae. (Prockop & Kivirikko 1984, 1995).

The 18-kb gene coding for the  $\alpha 1$  chain of type I collagen is located on the long arm of chromosome 17 (17q21.3-q22), and has 51 exons (Huerre *et al.* 1982, Chu *et al.* 1984). The 38-kb gene coding for the  $\alpha 2$  chain is located on the long arm of chromosome 7 (7q21.3-q22), and has 52 exons (de Wet *et al.* 1987). The size difference between the two genes reflects the differences in the sizes of the introns. The large majority of type I collagen is represented by a heterotrimeric molecule composed of  $\alpha 1(I)$  and  $\alpha 2(I)$  chains; two  $\alpha 1$  chains and one  $\alpha 2$  chain are coiled around each other in a characteristic triple helix. However, a very small proportion of type I collagen can be formed by three  $\alpha 1$  chains. Both the  $\alpha 1(I)$  and  $\alpha 2(I)$  chains consist of a signal peptide, N- and C-terminal non-collagenous propeptides, N- and C-terminal telopeptides, and a long, helical, collagenous domain. The collagenous domain includes 1014 amino acids with 338 Gly-X-Y repeats. (Prockop & Kivirikko 1995, Rossert & de Crombrughe 1996).

The synthesis of the fibril-forming collagen molecules is a multi-step process that includes intracellular processing, extracellular events and fibrillogenesis. After transcription of the *COL1A1* and *COL1A2* genes, mature mRNAs are translated, and the resulting polypeptides undergo post-translational modifications in the endoplasmic reticulum before the assembly of the triple helix and its liberation in to the extracellular space. The signal peptides are cleaved from the chains in the lumen of the endoplasmic reticulum. Concurrently with this cleavage, proline and lysine in the Y-position of the Gly-X-Y repeats, and a few prolines in the X-position, are hydroxylated to form 4-hydroxyproline, hydroxylysine and 3-hydroxyproline, respectively, by prolyl 4-hydroxylase, lysyl hydroxylase and prolyl 3-hydroxylase. In addition, either galactose, or both galactose and glucose, are added to the hydroxyl (OH) group of some of the hydroxylysine residues by galactosyltransferase and glucosyltransferase. Following the addition of a mannose-rich oligosaccharide to the C-propeptide of each pro $\alpha$  chain, the C-propeptide from two  $\alpha 1$  chains and one  $\alpha 2$  chain associate with each other through the formation of intra- and interchain disulfide bonds. This association promotes the formation of a C-terminal triple helix, which is subsequently extended towards the N-terminal in a zipper-like manner. After secretion of the procollagen into the extracellular space, the N-propeptides are cleaved by a procollagen N-proteinase and the C-propeptides by a procollagen C-proteinase. The collagen molecules then spontaneously assemble into quarter-staggered fibrils. Finally, during fibrillogenesis, some lysine and hydroxylysine residues are deaminated by a lysine oxidase, yielding aldehyde derivatives that form interchain cross-links. (Prockop & Kivirikko 1984, 1995, Engel & Prockop 1991, Kivirikko 1993, Myllyharju & Kivirikko 2004).

### 2.1.1.3 *Non-collagenous proteins*

About 10% of the organic matrix is composed of well over 200 proteins non-collagenous proteins, of which bone matrix proteoglycans and glycoproteins are the most abundant. Proteoglycans are formed by the covalent attachment of long-chain polysaccharides, glycosaminoglycans (GAGs), to the core protein molecules. Different subclasses of proteoglycans are generally characterized by the nature of the GAGs, which are composed of repeating carbohydrate units with varying degrees of sulfatation. Proteoglycans with protein cores composed of leucine-rich repeat sequences, such as decorin, biglycan, fibromodulin and osteoadherin, are the predominant forms found in the mineralized matrix (Fisher *et al.* 1989, Oldberg *et al.* 1989, Wendel *et al.* 1998). The hyaluronan-binding forms, in particular versican, are present during the early stages of osteogenesis (Robey 1996, Heinegård *et al.* 2002).

The number of glycoproteins identified in the bone matrix grows every year. These glycoproteins, such as alkaline phosphatase, osteonectin, Arg-Gly-Asp (RGD)-containing proteins and tetranectin, are produced at different stages of osteoblastic maturation. Proteins containing the RGD sequence include osteoadherin, thrombospondin, fibronectin, vitronectin, osteopontin and bone sialoprotein (Grzesik & Robey 1994, Sommarin *et al.* 1998). The RGD unit confers the ability to bind to the integrin class of cell surface receptors, and therefore these proteins can also be called cell attachment proteins. The functions of the glycoproteins include the control of cell proliferation, cell-matrix interactions, and the mediation of hydroxyapatite deposition (Robey 1996).

Other major constituents of bone matrix are the gla proteins, that include the matrix gla protein, osteocalcin (bone gla protein) and protein S. Osteocalcin and matrix gla protein are both members of a large family of mineral-binding, extracellular matrix proteins and are considered as skeletal gla proteins. They are synthesized by the osteoblasts and the chondrocytes, respectively (Hale *et al.* 1988, Hauschka *et al.* 1989). Other gla proteins, such as protein S, are not considered as skeletal proteins, because of their known functions in blood coagulation (Furie & Furie 1988, Ducy & Karsenty 1996).

### 2.1.1.4 *Mineral component*

Bone tissue provides a reservoir of essential minerals; about 99 % of the body calcium, 85 % of the phosphorus and 40-60 % of the body sodium and magnesium, are found within the bone crystals. These minerals can be released from the bone matrix via the process of bone resorption, and can be incorporated into the bone matrix during bone mineralization. The principal mineral component of bone is in the form of an inorganic calcium compound called hydroxyapatite, made up of calcium ions, phosphate ions and hydroxyl ions in the ratio  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ . (Hughes 2000).

## 2.1.2 *Bone remodelling and mineralization*

Bone remodelling is an important process that occurs throughout growth and in adult life. The balance between osteoclastic bone resorption and osteoblastic bone formation is

responsible for the maintenance of local bone mass in adults in areas known as basic multicellular units (BMU). The remodelling cycle consists of bone resorption by osteoclasts, resulting in the elimination of an area of bone matrix, followed by resynthesis of bone matrix by osteoblasts. The process is divided into four distinct phases: activation, resorption, reversal and formation.

During the activation phase, osteoclast precursors are recruited to sites of bone remodelling from the bone marrow, or from the blood. The systemic activation of bone remodelling is attributed to the actions of growth hormone, and thyroid and parathyroid hormones, while calcitonin and cortisone have a systemic inhibitory effect. The activation process involves complex interactions between multiple factors, including osteoclast activation factors released from the bone matrix by the resorbing osteoclasts, inducing osteoblast proliferation and differentiation, and locally-produced factors appropriate for the stimulation of all of these processes. (Marks & Hermey 1996, Schenk *et al.* 2002).

During the resorption phase, a quantum of bone matrix is destroyed by osteoclasts. The osteoclasts resorb the organic and inorganic components of bone, forming resorption lacunae (Blair *et al.* 1986). In cortical bone, the osteoclasts resorb the bone matrix following the long axis of the bone. In cancellous bone, the osteoclasts resorb the bone towards its center, forming lacunae, or cavities. (Eriksen *et al.* 1986).

During the reversal phase, lasting about nine days, osteoclasts undergo apoptosis and are replaced by osteoblasts (Eriksen 1986). It is during this phase that the bone resorption in the BMUs is coupled to bone formation. The reversal phase is followed by a formation phase, during which osteoblasts refill the resorption canal with lamellar osteoid. In cortical bone, the refilling of the resorption canal continues until the new osteons reach an outer diameter of about 200-250  $\mu\text{m}$ . In cancellous bone, osteoblasts fill the resorption canal in the trabecular surface, which has a diameter of 0.5-1 mm and an average depth of 50  $\mu\text{m}$ , forming a bone structural unit (BSU). (Schenk *et al.* 2002).

Mineralization begins about 13 days after osteoid has been formed. During this phase, the remaining osteoblasts on the bone surface become inactive lining cells, and the newly formed osteoid undergoes gradual mineralization. The main mineral component of bone is an imperfectly crystalline hydroxyapatite,  $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$ , which comprises about 1/4 of the volume, and 1/2 of the mass, of normal adult bone. (Eriksen *et al.* 1986, Marks & Hermey 1996, Schenk *et al.* 2002).

## 2.2 Bone disorders

### 2.2.1 Osteogenesis imperfecta (OI)

Osteogenesis imperfecta (OI) is a generalized disorder of connective tissue rich in type I collagen, such as bone, skin, ligaments, fascia, tendons, sclera and ears. The main manifestation of OI is bone fragility. Other manifestations in this phenotypically heterogeneous condition are blue sclera, hearing loss, dentinogenesis imperfecta (DI), joint hypermobility, hernias and easy bruising of skin (Sillence 1988). In almost all cases, OI results from mutations in one of the two genes (*COL1A1*, or *COL1A2*) encoding the  $\alpha$  chains of type I collagen. The first mutations were detected in the early 1980s (Chu *et al.* 1983, Pihlajaniemi *et al.* 1984, Barsh *et al.* 1985).

OI has been estimated to affect more than 1 in 10,000 individuals, although the precise incidence is difficult to evaluate, due to unfamiliarity with the disease and poor reporting of some of its forms (Byers & Cole 2002).

OI, a heterogeneous disorder, is divided into four main types, based on radiographic, genetic and clinical criteria (Sillence *et al.* 1979). The classification has subsequently been modified on a number of occasions (Glorieux *et al.* 2000, 2002), but it can be difficult to define the borders between the different types of OI, or to determine the exact OI type manifested in young patients.

OI type I is the mildest and most common form of dominantly inherited brittle-bone disease. Typically, affected individuals have blue sclerae, normal teeth, a normal or slightly short stature, and bone fragility ranging from mild to severe (Byers & Cole 2002). Fractures may affect any bone, but it is common to observe involvement of long bones, the ribs and the small bones of the hands and feet. Other manifestations are joint hypermobility, easy bruising of the skin, a triangular face, presenile hearing loss, or DI (Sillence *et al.* 1979). OI type I is subdivided on the basis of the absence (IA), or presence (IB) of DI (Levin *et al.* 1978). The incidence of OI type I has been estimated at between 1:15,000 and 1:20,000. However, due to the relatively mild presentation of the disease, the incidence may be underestimated. (Byers & Cole 2002).

OI type II is characterized by an extremely severe bone fragility that leads to death *in utero*, or within the first months of life (Sillence *et al.* 1984). Affected individuals have characteristic skeletal changes, including short extremities, bowed legs, abnormal ribs, flexed and abducted hips, and a very small thoracic cavity. At birth, the characteristic facial features include dark sclerae, a beaked nose and soft calvarium. Sillence has divided the OI type II into three subgroups based on clinical and radiographic findings. The incidence of this lethal OI type has been estimated to be 1:60,000 infants (Sillence *et al.* 1979). Originally, OI type II was suggested to be an autosomal recessive condition (Sillence *et al.* 1979), but it was later shown to be mostly autosomal dominant (Young *et al.* 1987, Byers *et al.* 1988).

The progressive deforming variety of OI type III is most likely genetically heterogeneous. OI type III is characterized at birth by a short stature, deformities resulting from *in utero* fractures, blueish sclerae and dentin abnormalities. If there is no evidence of fractures at birth, they usually appear during the next two years. Individuals with this disease have the highest fracture frequency of all OI types, and 200 fractures during a lifetime are not uncommon. Short stature is characteristic of OI type III, and an adult height of between 92 cm and 108 cm is typical. Sclerae are often pale blue at birth and generally become normal with age. Hearing loss is thought to be uncommon. The prevalence has been estimated at about 1:200,000 infants (Sillence *et al.* 1979). The autosomal dominant form of OI type III is the most common, but a recessive mode of inheritance has also been described. (Kuivaniemi *et al.* 1997, Byers & Cole 2002).

OI type IV is a dominantly inherited disorder characterized by normal, or grayish sclerae, mild to moderate deformity and, frequently, a short stature and DI. The presenting features include fractures occurring either *in utero*, during delivery, or during the newborn period. Fracture frequencies vary with age, but they usually decrease after the onset of puberty (Sillence *et al.* 1981). Although birth length is frequently normal, height is generally below the 25<sup>th</sup> percentile by two years of age, and frequently below the 5<sup>th</sup> percentile. Thereafter, the growth follows the lower percentile tracks. Other manifestations include joint hypermobility and scoliosis, the latter occurring in about one-

third of individuals and ranging from mild to severe (Hanscom & Bloom 1988). Hearing loss is supposed to be less common than in OI type I (Byers & Cole 2002).

In addition to the four traditional clinical OI types, two new OI types, V and VI, have been described. OI type V patients have a history of a moderate to severe fragility of long bones and vertebral bodies. Other characteristics typical of OI type V include calcifications of the interosseous membrane in the forearm, and hyperdense metaphyseal bands. OI type VI was first described in patients that had initially been diagnosed as having OI type IV. Type VI OI is a moderate to severe form of brittle-bone disease, with accumulation of osteoid due to a mineralization defect. Patients with OI type VI frequently sustain fractures and all have vertebral compression fractures. (Glorieux *et al.* 2000, 2002).

### 2.2.1.1 Hearing loss in OI patients

The hearing loss associated with OI usually begins during the second, or third decade of life. Population studies have revealed progressive hearing loss in up to 58% of adult OI patients (Pedersen 1984, Stewart & O'Reilly 1989). The progressive hearing loss often proceeds from a conductive hearing loss, to a mixed and sensorineural type (Pedersen 1984, Garretsen *et al.* 1997). Mixed hearing loss has been reported as the most frequent form in OI (Garretsen *et al.* 1997). Hearing loss is generally due to middle and inner ear pathology. The auditory ossicles of the middle ear, which include the malleus, incus and stapes, consist mainly of type I collagen, but type II collagen has also been observed in the stapes (Birchall *et al.* 1982, Yoo *et al.* 1988). Besides type II collagen, five other collagen genes are expressed in the vertebrate inner ear, including *COL1A2* (Heller *et al.* 1998). It is feasible that fractures of the auditory ossicles, or of the inner ear, can occur repeatedly during life in at least some forms of OI and, thus, contribute to the progressive hearing loss.

The pathological changes observed in the conductive hearing loss include a functional discontinuity of the ear ossicles, due either to atrophy and/or fractures of the stapedial crura, combined with thickening and fixation of the stapes footplate (Dieler *et al.* 1997). In contrast, cochlear hair cell loss, stria vascularis atrophy and calcification, tectorial membrane distortion and perilymph hemorrhage are autopsy findings that could account for sensorineural hearing loss. In addition, the histopathological findings of the otic capsule, thin walls of the middle ear, and of the ossicles have provided evidence for both deficient and abnormal ossification. Microfractures have also been found in the otic capsule and in the malleus. (Bergstrom 1981, Berger *et al.* 1985).

### 2.2.2 Osteoporosis-pseudoglioma syndrome (OPPG)

Osteoporosis-pseudoglioma syndrome (OPPG) (Robinow 1985, Brude 1986, Superti-Furga *et al.* 1985) was initially identified as an ocular form of OI (Beighton *et al.* 1985). OPPG is an autosomal recessive disease characterized by low bone mass, childhood fractures and abnormal eye development (Gong *et al.* 1996). In contrast to other heritable childhood disorders affecting bone mass, OPPG patients do not have defects in collagen synthesis, anabolic and catabolic hormones, calcium homeostasis, endochondral growth,

or bone turnover (Gong *et al.* 1996). Histological analyses of bone biopsies demonstrate a deficient trabecular bone volume, but a normal surface density and a normal presence of osteoblasts and osteoclasts on bone surfaces (Gong *et al.* 2001). In addition to the abnormal bone phenotype, individuals with OPPG have eye involvement. Many patients with OPPG are born with a severely disrupted ocular structure, phthisis bulbi, and persistent hyperplasia of the primary vitreous (PHPV) has been observed in children with milder eye involvement. Gong and coworkers suspected that the expression of LRP5 by cells within the vitreal vasculature is responsible for the involution of the primary vitreal vasculature during fetal growth. The failure of this process leads to fibrosis and contracture of the vitreal vasculature. The fibrotic process causes traction on other ocular structures and affects vision. (Gong *et al.* 2001).

### ***2.2.3 Different conditions with increased bone density***

The balanced process of bone resorption and formation maintains the homeostasis of bone tissue, and the disturbance of this balance can result in several skeletal pathologies with increased bone density. In contrast to osteoporosis with its multifactorial character, the underlying molecular genetic causes of several conditions with increased bone density appear to be monogenic. The pathogenic mechanisms leading to increased bone density can be divided into three groups, characterized by decreased bone resorption, increased bone formation, or a disturbed balance between bone resorption and formation (Janssens & Van Hul 2002).

The osteopetroses are a heterogeneous group of conditions of increased bone density characterized by skeletal sclerosis resulting from an aberrant, osteoclast-mediated bone resorption. Three clinically distinct forms of osteopetrosis are recognized: an infantile malignant autosomal recessive form (Loria-Cortés *et al.* 1977), an autosomal recessive form caused by CA II deficiency (Whyte *et al.* 1980), and an adult benign autosomal dominant form (Bollerslev & Andersen 1988). The osteopetroses represent a spectrum of clinical variants because of the heterogeneity of genetic defects that result in osteoclast dysfunction. The underlying molecular genetic defects of these three osteopetroses are discussed in chapter 2.3.3. (Whyte 2002).

High bone mass (HBM) is a syndrome characterized by increased bone formation (Boyden *et al.* 2002). It is an autosomal dominant disease characterized by increased bone density and variable clinical features, including entrapment neuropathies, increased levels of alkaline phosphatase, a square jaw and torus palatinus. The diagnosis is made based on BMD measurements using dual-energy X-ray absorptiometry with a densitometer. In affected subjects, the age- and sex-adjusted bone density is about 4 SD above the population mean. Johnson and coworkers were the first to report a linkage between a genetic locus and a very high spinal bone mineral density in one family (Johnson *et al.* 1997). In this kindred, the spinal Z (BMD) was 5.54 in 12 affected individuals. (Boyden *et al.* 2002, Little *et al.* 2002).

Disorders with imbalanced bone formation and resorption include familial expansile osteolysis (FEO), Paget's disease of bone (PDB) and juvenile Paget's disease. PDB is a dominantly inherited metabolic bone disease, usually occurring in persons over 40 years of age. It is characterized by focal areas of increased bone turnover at specific sites throughout the skeleton (Klein & Norman 1995). Typical clinical features include bone

pain, deformity and an increased susceptibility to fracture. However, the majority of patients are asymptomatic. A variety of complications, such as vertebral compression fractures, pathological fractures, or degenerative arthritis, may occur, depending on both the affected skeletal sites and the overall extent of the disease. The diagnosis is primarily accomplished by roentgenographic evaluation of the skeleton in which osteolytic lesions are distinctive. The underlying pathophysiology of PDB appears to reflect a localized increase in the number of osteoclasts, with a subsequent increase in osteoblastic activity. Within the pagetic lesions, osteoclasts are large, multinucleated and overactive, and contain paramyxovirus-like inclusions. Mills and Singer (1976) were the first to suggest a viral etiology in PDB, but while the significance of this is still uncertain, there is evidence that genetic factors play a role in the pathogenesis of the disease. (Janssens & Van Hul 2002).

### ***2.2.4 Osteoporosis***

Osteoporosis is a multifactorial metabolic bone disease characterized by low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (Consensus Development Conference 1993). It is a complex disease with high social and economic costs, affecting about 30 % of post-menopausal white women in the USA, and the proportion rises to 70 % in women over the age of 80 years (Melton 1995). Osteoporosis is defined as a bone mineral density (BMD) of 2.5 SD, or more, below the average value for pre-menopausal women (T score < - 2.5 SD). Thus, the diagnosis of osteoporosis focuses on the assessment of BMD. The risk factors of osteoporosis are numerous, the most significant being low bone mass, age and female sex. In general, risk factor scores show poor specificity and sensitivity for the prediction of osteoporosis and fracture risk, and some risk factors vary significantly with age (Kanis 2002).

Both genetic and environmental factors have an influence on the development of low peak bone density. Peak bone mass is attained by late adolescence and is a major determinant of reduced bone density, which predisposes to osteoporotic fractures. Peak bone mass is under strong genetic control, and twin studies suggest that genetic factors account for up to 80 % of BMD variance in young adults (Pocock 1987). (Seeman 2002).

Post-menopausal osteoporosis is characterized by fractures at sites containing substantial proportions of both cortical and cancellous bone. After peak bone size and peak BMD have been reached, bone remodelling continues on the endosteal surface. Bone loss accelerates at menopause, because the remodelling rate increases as estrogen deficiency reduces the osteoblast lifespan and increases the osteoclast lifespan (Seeman 2003). Osteoblasts resorb the bone, leaving a focal resorptive cavity on the trabecular and endocortical surfaces, and then refill the cavity with new bone. When the volume of bone that has been resorbed is greater than that which is formed, the imbalanced remodelling results in thinning and, finally, the loss of trabecular connectivity (Seeman 2002). Up-regulation of bone resorption and down-regulation of bone formation are caused by the cumulative effects of a number of factors, such as normal aging, dietary calcium deficiency, estrogen deficiency and lower physical activity. (Riggs & Melton 1983, Bono & Einhorn 2003).

In addition to supplemental calcium and vitamin D, currently available treatments include therapeutic protocols that mainly decrease resorptive bone remodelling and, thus, retard bone loss in post-menopausal osteoporosis. They include: estrogen, selective estrogen-receptor modulators, calcitonin, calcitriol and bisphosphonates, such as alendronate. The bisphosphonates slow down the action of osteoclasts, increase the osteoblasts' lifespan, and decrease bone remodelling. The newest therapeutic agent is a synthetic form of parathyroid hormone (PTH), and it is the first osteoporosis drug that stimulates new bone formation. (Neer *et al.* 2001, Johnell *et al.* 2002, Seeman 2003, Mellstrom *et al.* 2004).

### ***2.2.5 Idiopathic juvenile osteoporosis (JIO)***

Osteoporosis is increasingly being recognized in children, usually as a complication of a chronic illness (Steelman & Zeitler 2001). Several other factors, such as medications, nutrient and hormone deficiencies and low physical activity, may also contribute to either reduced bone mass, or to bone quality impairment. Primary osteoporosis is less commonly recognized, and is usually diagnosed as either OI, or juvenile idiopathic osteoporosis (JIO). JIO of unknown etiology usually presents peri-pubertally as an acute symptomatic osteoporosis with bone pain and fractures, in an otherwise healthy child (Dent & Friedman 1965). Frequently observed radiological features include vertebral compression fractures; less frequent are osteopenia and fractures of the long bones. Histologically, the volume of cancellous bone is decreased and bone remodelling is disturbed by a very low bone formation. (Rauch *et al.* 2000, Lorenc 2002, Rauch *et al.* 2002).

### ***2.2.6 Rickets and osteomalacia***

Rickets in the growing child, or adolescent, and osteomalacia in the adult, develop in a variety of clinical situations and have in common an absence of, or delay in, the mineralization of growth cartilage and newly formed bone collagen. Osteomalacia and rickets are caused by a dietary vitamin D deficiency, deficiency of vitamin D metabolites, intestinal malabsorption, or renal disease. The malabsorption of vitamin D from the intestine is the most common cause of osteomalacia. In addition, an hereditary deficiency in  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> ( $1,25(\text{OH})_2\text{D}_3$ ) production causes vitamin D-dependent rickets (VDDR) type I, while hereditary defects of the vitamin D receptor-effector system causes vitamin D-dependent rickets type II. Thus, a deficiency in vitamin D action will lead to hypocalcemia, hypophosphatemia and hyperparathyroidism in rickets and osteomalacia. The clinical characteristics in adults include spontaneous incomplete fractures, bone pain and weakness of proximal limb muscles. In children, rickets leads to the indentation of the ribs at the diaphragmatic insertion, and a widening of the wrists. The characteristic histological features of rickets and osteomalacia include an inadequate mineralization of the organic matrix of bone, resulting in its reduced strength. The specific radiographic features of rickets reflect the failure of cartilage calcification in that metaphyses are widened, or cupped. The radiographic manifestations in osteomalacia

include generalized, or non-specific, osteopenia and pseudo-fractures. (Lieberman 1996, Hughes 2000).

### ***2.2.7 Stress fractures***

The first report of a stress fracture was made in 1855 by a military surgeon, who described soldiers with oedematous and painful feet (Breithaupt 1855). Two years after Wilhelm Röntgen discovered X-rays, in 1897, this condition was shown to be due to a fracture of the metatarsal shaft and was subsequently termed a "march fracture" (Schulte 1897). Over the past 25 years, stress fractures have been observed with increasing frequency in non-military populations, primarily due to remarkable increases in the participation in physical activities. Each year, many military recruits and athletes who start intensive physical training also experience the dramatic effects of a femoral neck stress fracture. The etiology of stress fractures is likely to be multifactorial, with a well-defined environmental component. A role has been suggested for genetic factors, but no susceptibility genes have yet been identified (Friedman *et al.* 2001).

Stress fractures are of two general types. The first, known as an insufficiency fracture, results from normal stress applied to abnormal bone. Underlying conditions include, for instance, PDB, osteomalacia, rickets and OI. The second type, known as a fatigue fracture, occurs when normal bone is subjected to repetitive stresses that lead to mechanical failure over time (Anderson & Greenspan 1996).

A typical symptom of stress fractures is a localized pain that gradually worsens, most commonly in the lower extremities. The pain is aggravated by physical activity and is relieved by rest. The diagnosis of bone stress fractures is based on the patient's history of increased physical activity and radiological findings, which can vary from normal, to evidence of a displaced fracture. (Anderson & Greenspan 1996, Shaffer 2001, Jones *et al.* 2002).

Stress fracture has been suggested to be a biological process during which increased mechanical usage stimulates bone turnover, resulting in focally increased bone remodelling and decreased bone mass. Continuous focal loading in transiently osteopenic bone leads to the accumulation of bone micro-damage and failure. Consequently, when mechanical loading is prolonged on a region of bone with high turnover, it leads to stress fracture. The association of remodelling and damage is supported by histological evidence indicating that micro-damage accumulation and the initiation of micro-damage repair by remodelling occur at the stress fracture site (Mori *et al.* 2001). A number of studies support the hypothesis whereby stress fractures occur in this manner, but there is little direct data on the pathophysiology of stress fractures. (Li *et al.* 1985, Mori & Burr 1993, Schaffler & Boyd 1997, Schaffler 2001).

## **2.3 Molecular genetics of bone disorders**

Most diseases are the result of a combined action of genes and the environment, and the relative importance of the role played by the genetic component may be either large, or small. Thus, disorders caused entirely, or partially, by genetic factors can be classified

into three main types: single-gene disorders, chromosomal disorders, or multifactorial disorders. The single-gene disorders can be caused by a mutation in only one allele, as in OI, or in both. In chromosomal inheritance, the defect is due to an excess, or a deficiency of the genes contained in whole chromosomes, or in chromosomal segments. Multifactorial disorders results from a combination of small variations in different genes, as well as environmental factors, as in the case of osteoporosis (Hall 1993).

### ***2.3.1 The role of type I collagen in osteogenesis imperfecta***

Over 90 % of patients with OI have been shown to have a mutation in the genes encoding type I collagen, *COL1A1* and *COL1A2*. Mutations in these genes are not present in OI types V and VI, so the absence of abnormalities in type I collagen does not always preclude the diagnosis of OI. (Sykes *et al.* 1990, Wallis *et al.* 1993, Glorieux *et al.* 2000, 2002).

Type I collagen gene mutations causing OI include single nucleotide substitutions, deletions, insertions, missense mutations, nonsense mutations, exon skipping and multi-exon rearrangements. Mutations in the type I collagen genes resulting in OI can be divided into two major categories; those that cause the synthesis of a structurally abnormal gene product, and those leading to a reduced synthesis of a gene product. Usually, mutations involving the substitution of a glycine residue lead to more severe OI phenotypes than mutations leading to decreased protein levels. However, identical mutations may result in phenotypes varying from mild to lethal. Thus, the relationship between pathogenic mutations and the disease phenotype is complex, and the underlying mechanisms are poorly understood. (Byers *et al.* 1991, Prockop *et al.* 1994, Wolf 1997, Byers & Cole 2002).

The most common mutations in OI patients involve substitutions of obligatory glycine residues, which appear as every third amino acid in the triple-helical domain of the pro $\alpha$  chain. It has been suggested that substitutions of glycine residues in the triple helix have the most severe phenotypic consequences when they occur towards the C-terminal of the molecule. In addition, the nature of the substituting amino acid and the local domain structure have been suggested to affect the resulting OI phenotype. In patients with the most debilitating forms of the disease, the mutations lead to the synthesis of structurally abnormal pro $\alpha$  chains, which exert their effects through three different molecular mechanisms. The first mechanism, called the dominant negative effect, or ‘‘procollagen suicide’’, occurs when the procollagen molecule contains both abnormal and normal pro $\alpha$  chains, and the abnormal chains prevent the folding of the protein into a triple-helical conformation, leading to the degradation of the molecule. In the second mechanism, the presence of abnormal pro $\alpha$  chains interferes with the cleavage of the N-propeptide from the protein. This results in thin, irregular fibrils, because the partly processed procollagen co-polymerises with normal collagen. In the third mechanism, the glycine substitution does not prevent the folding, but rather interferes with it, resulting in a conformational change in the molecule. Co-polymerization of mutated and normal collagen can lead to the assembly of abnormally branched, unusually thick and short collagen fibrils, delayed fibril formation and a reduced amount of total collagen

incorporated into the fibrils. (Byers *et al.* 1991, Engel & Prockop 1991, Kuivaniemi *et al.* 1991, Prockop *et al.* 1994, Kuivaniemi *et al.* 1997).

OI type I is usually caused by frameshift, nonsense, or splicing mutations that result in *COL1A1* haploinsufficiency. These mutations lead to the appearance of premature termination codons and nonsense-mediated mRNA decay (NMD), effectively producing a *COL1A1* null allele. Premature termination codons can be introduced into a gene by insertions and deletions that lead to shifts in the translational reading-frame, single base-pair changes that create nonsense codons, and single base-pair substitutions in conserved donor, or acceptor splice sites that lead to cryptic splicing and to retention of intronic sequences in the mature mRNA. Mutations in the *COL1A2* gene that result in haploinsufficiency have not been observed in OI patients. Interestingly, it was recently shown that the complete loss of the pro $\alpha$ 2 (I) chains does not associate with bone fractures, but rather causes a rare, recessively inherited, cardiac valvular form of Ehlers-Danlos syndrome. (Willing *et al.* 1992, 1994, 1996, Slayton *et al.* 2000, Schwarze *et al.* 2004).

The second most common mutations in OI are those located in the conserved RNA splice consensus sequences, which can lead to partial or complete exon exclusion, or to intron inclusion due to interrupted splicing (Byers *et al.* 1991). These RNA splicing mutations can cause either the synthesis of a structurally abnormal gene product, or a reduced synthesis of the gene product. The resulting phenotypes can vary from type I to type IV OI. Exonic mutations, such as nonsense, missense, or silent mutations, can also affect pre-mRNA splicing. These exonic mutations can lead to the disruption of cis-elements important for correct splicing and, thus, lead to exon skipping (Cartegni *et al.* 2002). Moreover, intronic mutations can either create, or activate cryptic splice sites, or disrupt cis-acting elements, and cause exon skipping (Faustino & Cooper 2003). However, this process has not been described in OI patients.

In addition to the above-mentioned mutations causing OI, there are more rare mutations types, including multi-exon rearrangements, in-frame deletions and insertions, and mutations in the C-terminal domain. Multi-exon rearrangements are lethal and uncommon. Short in-frame deletions and duplications represent a small percentage of the more than 300 reported OI-producing mutations. The phenotypes of the patients with these mutations are variable, and depend on whether the mutations have a dominant negative effect, or induce haploinsufficiency. Mutations in the C-terminal domain can result in a mild, or a lethal phenotype. It has been suggested that the mild effect is caused by exclusion of the mutated chain from the normal chains, resulting in a non-functional allele. In contrast, the lethal phenotype results from the inclusion of an abnormal chain into the procollagen molecules (Byers *et al.* 1991, Pace *et al.* 2001).

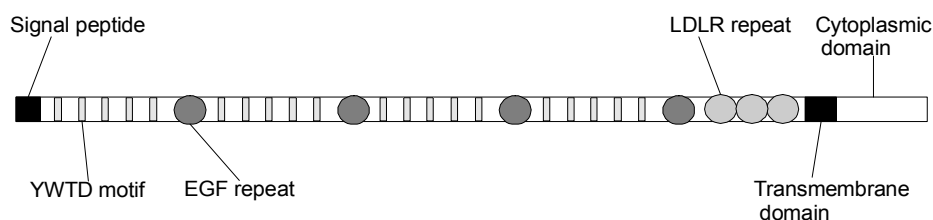
### ***2.3.2 The role and function of LRP5 in bone disorders***

#### ***2.3.2.1 Structure of LRP5 protein***

The low-density lipoprotein (LDL) receptor related protein 5 (*LRP5*) gene, which maps to chromosome 11q12-13 in humans, encodes a transmembrane protein of 1,615 amino acids that is a member of the LDL receptor-related family (Hey *et al.* 1998). The low-density

lipoprotein receptor (LDLR) family comprises cell surface receptors involved in diverse biological processes, including lipid metabolism, retinoid uptake and neuronal migration. These proteins are involved in the receptor-mediated endocytosis of specific ligands (Krieger & Herz 1994), which can either be a lipoprotein, or a protein. The low-density lipoprotein receptor protein is the prototype of the LDLR family. The *LRP5* and *LRP6* genes represent a new class of the LDLR family based upon analysis of the proteins they encode.

The extracellular domain of LRP5 (Figure 1) begins with a putative signal peptide and a series of four epidermal growth factor (EGF) repeats with an associated spacer domain, which is itself composed of five repeated units of approximately 50 amino acids residues, each having a tyrosine-tryptophan-threonine-aspartate (YWTD) motif (Hey *et al.* 1998). Generally, the position and sequence of the YWTD motifs are highly conserved in the LRP5 and LRP6 proteins (Brown *et al.* 1998). A cluster of three consecutive LDLR repeats is adjacent to the fourth EGF repeat. The LDLR repeats are approximately 40 amino acids in length and contain six conserved Cys residues, as well as a serine-aspartic acid-glutamic acid (SDE) motif as a characteristic feature. LRP5 and LRP6 both have a unique organization of EGF and LDLR repeats compared to those of the other LDLR family members (Brown *et al.* 1998). A single transmembrane domain of 23 amino acids follows the LDLR repeats (Hey *et al.* 1998).



**Fig. 1. Structure of the LRP5 protein. The extracellular domain of LRP5 begins with a signal peptide and four repeated units, each consisting of an epidermal growth factor (EGF) repeat and five YWTD motifs.**

The cytoplasmic domain of LRP5 bears no homology to that of the other LDLR family proteins either. The cytoplasmic domain of LRP5 is rich in Pro and Ser residues, with 16% Pro and 15% Ser (Brown *et al.* 1998). LRP5 does not have the conserved NPXY motif located in the cytoplasmic domain of most members of the LDLR family, but instead has five copies of PPP(S/T)P motifs (Chen *et al.* 1990, Tamai *et al.* 2004).

The *LRP6* gene is most closely related to the *LRP5* gene. The cDNA sequences of *LRP5* and *LRP6* have 64% identity over 5110 nt, and their protein coding sequences of 1615 and 1613 aa are 71% identical (Brown *et al.* 1998).

### 2.3.2.2 The role of LRP5 in Wnt/ $\beta$ -catenin signalling

*LRP5* and its closely related homologue, *LRP6*, are similar to the drosophila *arrow* gene, which is required for signalling of genes in the wingless, or Wnt, family of genes in fruit flies (Wehrli *et al.* 2000). LRP5 and LRP6 play the same role in vertebrates, acting as co-receptors for Wnt proteins, that comprise a family of secreted growth factors with critical

roles at almost all stages of development, throughout evolution. In contrast to LRP6, our understanding of the role of LRP5 in Wnt signalling is limited (Tamai *et al.* 2000, Kato *et al.* 2002).

The interaction of Wnt proteins with their receptors on the cell surface is the first step in transducing the extracellular signal into intracellular responses (Figure 2). Wnt proteins bind frizzled (Fz), a membrane protein with seven transmembrane domains. In addition to Fz proteins, the canonical Wnt/ $\beta$ -catenin signalling pathway requires LDLR-related proteins, which, in vertebrates, are represented by LRP5 and LRP6 (Pinson *et al.* 2000, He *et al.* 2004). It has been suggested that Wnt may induce the formation of a Fz-Lrp5/6 complex, which triggers downstream signalling (Tamai *et al.* 2000). *In vitro* studies have shown that dickkopf-1 (Dkk-1) antagonizes Wnt/ $\beta$ -catenin signalling by binding to LRP5/6 (Mao *et al.* 2001a).

The intracellular domain of LRP5 binds axin; a scaffolding protein that contains binding sites for adenomatous polyposis coli (Apc), glycogen synthase kinase 3 (Gsk3), casein kinase 1 (Ck1),  $\beta$ -catenin, and possibly other proteins (He *et al.* 2004). The binding of the LRP5 intracellular domain to axin allows the Wnt co-receptors to control  $\beta$ -catenin phosphorylation and degradation (Mao *et al.* 2001b). In the presence of Wnt proteins,  $\beta$ -catenin phosphorylation and degradation is inhibited. Accumulation of  $\beta$ -catenin forms a nuclear complex with the DNA-bound T cell factor/Lymphoid enhancer factor (TCF/LEF) transcription factors and, together, they activate Wnt-responsive genes (He *et al.* 2004). Without Wnt proteins, axin, Apc, Gsk3, Ck1 and  $\beta$ -catenin form a complex, in which  $\beta$ -catenin is phosphorylated by Gsk3, leading to  $\beta$ -catenin degradation. As  $\beta$ -catenin is degraded, the formation of an active signalling complex does not occur (Mao *et al.* 2001b, He *et al.* 2004). Since LRP5 affects the accrual of bone mass by Wnt-mediated osteoblastic proliferation and differentiation, normal osteoblastic proliferation and differentiation processes are disturbed when the active signalling complex is not formed (Gong *et al.* 2001).

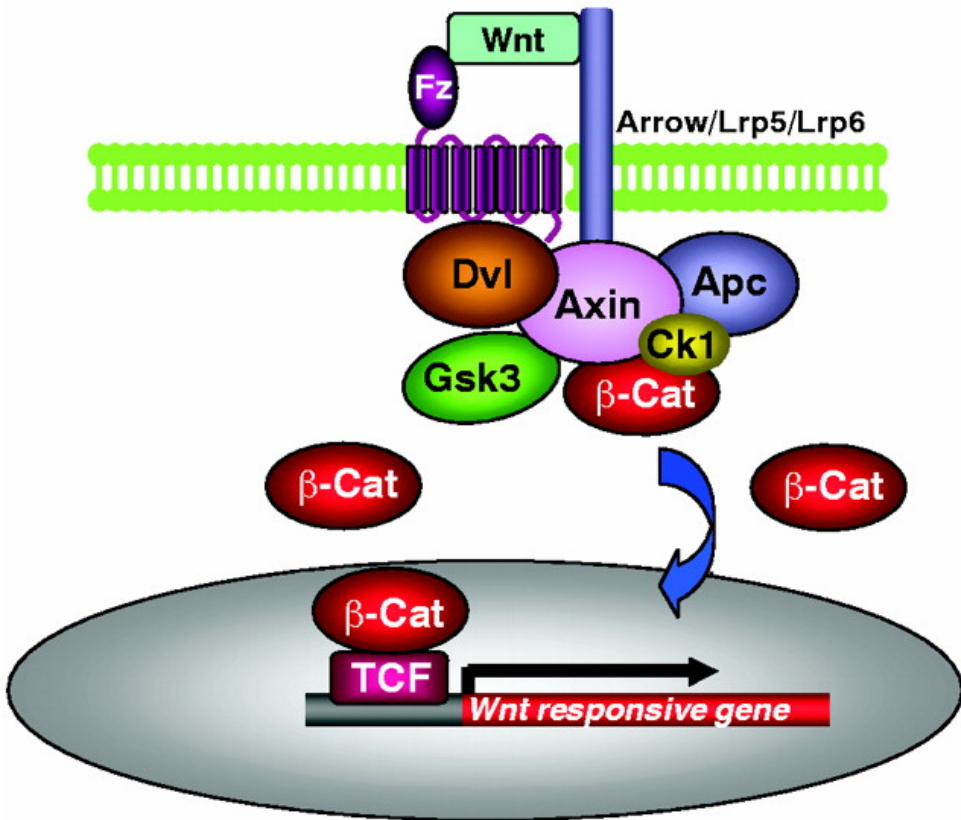


Fig. 2. Schematic representation of normal Wnt/ $\beta$ -catenin signalling in the presence of Wnt protein (Modified from He *et al.* 2004).

### 2.3.2.3 *LRP5* and bone disorders

Several recent studies point to an important role of the *LRP5* gene in various bone disorders. Loss-of-function mutations in *LRP5* are responsible for OPPG, whereas *LRP5* gain-of-function mutations cause high-bone-mass syndromes (Boyden *et al.* 2002, Little *et al.* 2002, Van Wesenbeeck *et al.* 2003). More recently, it was suggested that polymorphisms in the *LRP5* gene are BMD determinants and contribute to the risk of developing osteoporosis (Ferrari *et al.* 2004, Mizuguchi *et al.* 2004). However, it is not clear how these different polymorphisms and mutations affect the function, or expression, of the LRP5 protein.

Gong and coworkers (2001) identified six homozygous, disease-causing, frameshift and nonsense mutations in individuals with OPPG. Putative disease-causing homozygous missense and heterozygous nonsense, frameshift and missense mutations were also observed in affected patients from non-consanguineous families. Interestingly, carriers of *LRP5* mutations had a lower bone mass than non-carriers, indicating that the effects of

this gene are dominant for the regulation of bone mass. The mechanism of action of the OPPG disease-causing mutations in the *LRP5* gene is loss-of-function. Gong and coworkers (2001) showed that LRP5 is able to mediate Wnt signalling *in vitro* via the canonical pathway. They further showed that LRP5 is required for the proliferation of osteoblasts. These results indicated that LRP5 affects the accrual of bone mass by Wnt-mediated osteoblastic proliferation and differentiation. In addition to bone phenotype, OPPG patients suffer from a severe disruption of ocular structures that has been suggested to result from a failed regression of the primary vitreal vasculature during fetal growth (Gong *et al.* 2001). This phenotype was also observed in *Lrp5*<sup>-/-</sup> mice, possibly due to a lack of capillary endothelial apoptosis in the eye. Lack of apoptosis may be due to defects in ocular macrophages, which express *Lrp5* and are required for the induction of capillary cell death (Gong *et al.* 2001, Kato *et al.* 2002).

In an effort to identify genes that play a role in regulating BMD, Johnson and coworkers reported the linkage of a gene causing HBM in a single family (Johnson *et al.* 1997). Linkage analysis showed that the trait was localized to a 30-cM region of 11q12-13 with a lod score of 5.74. Later, Little and coworkers identified a heterozygous G171V mutation in the *LRP5* gene in one family with non-syndromic HBM. This mutation occurs near the fourth YWTD repeat of the first YWTD/EGF domain in LRP5 (Little *et al.* 2002), and is highly conserved from drosophila to man. Boyden and coworkers found the same G171V mutation in patients with syndromic HBM (Boyden *et al.* 2002). In addition, they showed that this gain-of-function mutation impairs the action of dickkopf-1 (Dkk-1), an antagonist of the Wnt pathway, thus increasing Wnt signalling. This was supported by the fact that the levels of fibronectin, a known target of Wnt signalling, were elevated in patients with this mutation. Further evidence to support the importance of *LRP5* in regulating bone mass was obtained from transgenic mice expressing the same G171V mutation. These mice had a similar phenotype, with high bone mass and enhanced bone strength. (Boyden *et al.* 2002, Babij *et al.* 2003).

Six novel missense mutations have also been found in patients with an increased bone density, including endosteal hyperostosis, Van Buchem disease, autosomal dominant osteosclerosis and autosomal dominant osteopetrosis type I (Van Wesenbeeck *et al.* 2003).

### ***2.3.3 Molecular genetic defects in disorders with increased bone density***

Defects in bone resorption can give rise to conditions with an increased bone density. The resorption of mineralized bone tissue is a process involving the dissolution of bone mineral and the enzymatic degradation of the organic bone matrix. For both processes, an acidic environment is needed, and it is created in the extracellular compartment located between the osteoclasts and the bone surface. Defects in the acidification process of this extracellular compartment lead to osteopetrosis. The underlying molecular genetic defects have been identified in four forms of osteopetrosis. Firstly, a mutation in the carbonic anhydrase II (*CA II*) gene leads to an autosomal recessive form of osteopetrosis (Sly *et al.* 1983). This enzyme catalyses the formation of protons in the cytoplasm of the osteoclasts; CO<sub>2</sub> and H<sub>2</sub>O are converted to HCO<sub>3</sub><sup>-</sup> and protons (H<sup>+</sup>). Secondly, loss-of-function mutations in the *ATP6t* gene, encoding the osteoclast-specific 116-kD subunit of the vacuolar proton pump (vacuolar H<sup>+</sup>-ATPase), are present in an autosomal recessive form of malignant osteopetrosis (Kornak *et al.* 2000). The vacuolar H<sup>+</sup>-ATPase shuttles

the protons, formed by the action of CA II, to the extracellular compartment. Together with the Cl<sup>-</sup> ions, transported by the chloride channel CLCN7, the acidity of the extracellular compartment needed for the demineralisation process is established. Mutation of the *CLCN7* gene can lead to autosomal dominant osteopetrosis type II (Cleiren *et al.* 2001), while mutations in the *LRP5* gene can lead to autosomal dominant osteopetrosis type I. (Janssens & Van Hul 2002, Van Wesenbeeck *et al.* 2003).

A disturbed balance between bone formation and resorption can give rise to conditions with an increased bone density, such as familial expansile osteolysis (FEO), Paget's disease of bone (PDB) and juvenile Paget's disease. Mutations in the OPG/RANK/RANKL pathway have been associated with these clinically related conditions. The clinical similarities are explained by the same pathogenetic mechanism involving increased OPG/RANK/RANKL signalling. Firstly, activating mutations in the *TNFRSF11A* gene, encoding the receptor activator of NF $\kappa$ B (RANK), causes FEO (Hughes *et al.* 2000). Upon interaction with RANKL, RANK, a transmembrane receptor on the osteoclast membrane, induces the OPG/RANK/RANKL pathway, thus stimulating the differentiation and activation of osteoclasts. Secondly, activating mutations in the *SQSTM1/p62* gene cause Paget's disease of bone (PDB) (Laurin *et al.* 2002). The p62 protein has several divergent functions, one of which is a downstream effector of the RANK/RANKL regulatory system, leading to the upregulation of the OPG/RANK/RANKL signalling pathway. The activating mutations in the *p62* gene thus lead to increased bone resorption compensated by increased formation. Finally, loss-of-function mutations in the *TNFRSF11B* gene, encoding osteoprotegerin (OPG), cause juvenile Paget's disease (Whyte *et al.* 2002). Normally, OPG can block the RANK/RANKL interaction system. The loss-of-function mutations in the *TNFRSF11B* gene have an activating effect on the OPG/RANK/RANKL pathway. (Hughes *et al.* 2000, Janssens & Van Hul 2002, Laurin *et al.* 2002, Whyte *et al.* 2002).

### 2.3.4 Candidate genes in osteoporosis

Osteoporosis is a multifactorial disease that is influenced by both environmental and genetic factors. Much is known about the factors that regulate bone turnover and about the proteins that make up normal bone matrix. This has facilitated studies of candidate genes responsible for osteoporosis. The common form of osteoporosis is generally considered to be multifactorial, arising from the interaction of common polymorphic alleles with multiple environmental factors (McGuigan *et al.* 2002). Even though osteoporosis can be one of the findings in monogenetic diseases, a monogenetic mode of inheritance cannot be confirmed in non-syndromic osteoporosis. (Smith *et al.* 1994, Gong *et al.* 2001, Peacock *et al.* 2002).

Studies of candidate genes for osteoporosis have focused on cytokines, growth factors that regulate bone turnover, genes that encode components of bone matrix, and genes that encode receptors for calciotropic hormones (Ralston 2002), yielding more than 200 potential candidates. The most important candidate genes are presented below (Table 1).

The first association study was made between the  $\alpha_2$ -HS-glycoprotein (*ASHG*) gene and bone mass.  $\alpha_2$ -HS-glycoprotein is present in the bone matrix and functions as an immunoregulator (Dickson *et al.* 1994).

The estrogen receptors (ESRs) are responsible for the actions of estrogenic steroids on their target tissues, which include skeletal tissues. The interaction of estrogen with its receptor regulates bone turnover and skeletal growth, thus playing an essential role in maintaining bone mass in women (Lindsay 1998). The *ESR1* and *ESR2* genes code for ESR1 and ESR2, respectively. Polymorphisms in the *ESR1* gene have been most widely studied, but no consistent associations between the *ESR1* polymorphisms and bone mass have been observed (Peacock *et al.* 2002).

*Table 1. Candidate genes in osteoporosis and functions of the gene products*

Category	Candidate gene	Protein	Function
Calcitropic hormones and receptors	<i>VDR</i>	VDR	Bone cell differentiation, bone turnover, calcium absorption
	<i>ESR1</i>	ESR1 ( $\alpha$ )	Osteoclast-osteoblast activity
	<i>ESR2</i>	ESR2 ( $\beta$ )	Osteoclast-osteoblast activity
	<i>PTH</i>	Parathyroid hormone	Calcium homeostasis; osteoclast-osteoblast activity
	<i>PTHRI</i>	Parathyroid hormone receptor	Chondrocyte differentiation
	<i>CTR</i>	Calcitonin receptor	Osteoclast function
Cytokines, growth factors and receptors	<i>TGFBI</i>	TGF $\beta$	Osteoclast-osteoblast activity
	<i>IGF1</i>	IGF-1	Growth-promoting effects
	<i>IL6</i>	IL-6	Osteoclast differentiation and activity
Bone matrix	<i>COL1A1</i>	Type I collagen	Matrix component
	<i>BGLAP</i>	Osteocalcin	Matrix component
	<i>AHSG</i>	$\alpha_2$ HS-glycoprotein	Matrix component
	<i>MMP</i>	Collagenase	Resorption of bone matrix

The genes encoding type I collagen (*COL1A1* and *COL1A2*) are important candidates for the pathogenesis of osteoporosis, since type I collagen is the main structural protein of bone. Polymorphisms in the coding regions of these genes are rare and do not appear to be associated with osteoporosis (Spotila *et al.* 1994). The most interesting polymorphism of the *COL1A1* gene is located in intron 1, at the binding site for the Sp1 transcription factor. It has been reported to account for part of the variance in BMD in the normal population and to be associated with fragility fractures (Grant *et al.* 1996, Uitterlinden *et al.* 1998). The mechanism by which the Sp1 polymorphism predisposes to osteoporosis has been suggested to be due to the "s" allele, which increases the affinity for Sp1 protein binding and is associated with an elevated, allele-specific, transcription in heterozygotes carrying this allele (Mann *et al.* 2001). Later, a meta-analysis performed by Mann and Ralston (2003) indicated that *COL1A1* Sp1 alleles are associated with a modest reduction in BMD and a significant increase in the risk of osteoporotic fractures, particularly of the vertebrae.

Polymorphisms of several other candidate genes, including those encoding bone structural proteins and regulatory proteins or hormones involved in bone metabolism, have been associated with bone mass and/or osteoporotic fractures (Table 1). Other genes, such as those of the androgen receptor (*AR*), apolipoprotein E (*apoE*) and the

interleukin-1 receptor antagonist (*IL-1RN*), have also been associated with osteoporosis (Steward & Ralston 2000). The role of the vitamin D receptor (*VDR*) as a candidate gene for osteoporosis is discussed in chapter 2.3.5.2.

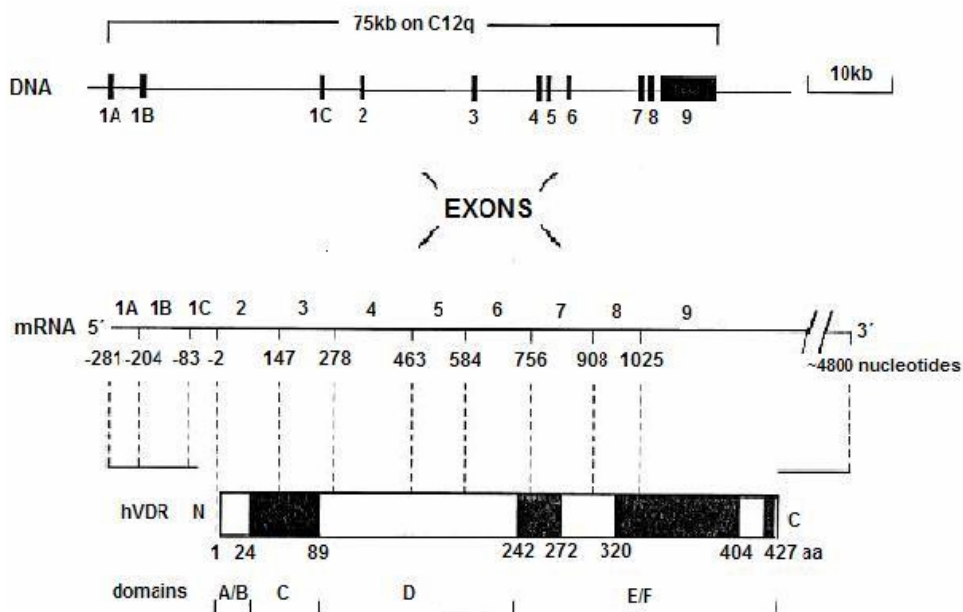
### ***2.3.5 The role of VDR in bone disorders***

#### ***2.3.5.1 Structure and function of VDR***

The *VDR* gene, which encodes the vitamin D receptor, consists of 11 exons and spans approximately 75 kb (Miyamoto *et al.* 1997). Its chromosomal location is 12q12-q14 (Faraco *et al.* 1989, Szpirer *et al.* 1991). The non-coding 5'-end of the *VDR* gene includes exons 1A, 1B and 1C, while its translated product is encoded by exons 2 to 9. Three unique mRNA isoforms are produced by alternative splicing of exons 1B and 1C. A second in-frame ATG codon in codon 4 of the *VDR* gene, arising from a T to C conversion, provides a second potential translation start site resulting in the production of a shortened protein of only 424 amino acids (Figure 3). (Miyamoto *et al.* 1997).

VDR has been recognized as a member of the superfamily of nuclear receptors that includes the steroid and thyroid hormone receptors that regulate gene expression in a ligand-dependent manner. VDR is more closely related to the thyroid hormone receptors. The functional regions of VDR are designated as A/B, C, D and E/F (See Figure 3). The A/B domain is known as the transactivation domain, and is poorly conserved in both amino acid composition and in size. The function of this region has not been clearly defined. The DNA-binding domain, C, is the best conserved containing two zinc finger-like motifs encoded by exons 2 and 3. The D domain is the hinge region between the DNA-binding domain and the ligand-binding domain. The E/F domain is the conserved steroid-binding region responsible for ligand binding, dimerization and transcriptional activation. (Krust *et al.* 1986, Norman & Collins 1996, Miyamoto *et al.* 1997).

VDR plays a central role in the biology of vitamin D. It binds specifically to an active form of vitamin D,  $1,25(\text{OH})_2\text{D}_3$ , and interacts with target-cell nuclei to produce a variety of biological effects. VDR is a  $1,25(\text{OH})_2\text{D}_3$ -dependent transcription factor that controls gene expression by heterodimerizing with retinoid X receptors (RXRs) and associating specifically with vitamin D response elements (VDREs) in target genes (MacDonald *et al.* 1993). The hormone-receptor complex induces calcemic and phosphatemic effects in bone, intestine, kidney and the parathyroid, that result in normal bone mineralization and remodelling. VDR also participates in the differentiation of skin cells and in the normal skin hair growth cycle. VDR also has a myriad of different bioactivities in immune, neural, epithelial, and endocrine tissues and organs. (Haussler *et al.* 1998).



**Fig. 3.** The structural organization of the human *VDR* gene locus including 11 exons (1A, 1B, 1C, and 2 through 9). A 10-kb scale-bar is indicated on the right. The location of exons relative to the mRNA transcript of ~4800 nucleotides (mRNA), and the encoded VDR protein of 427 amino acids (hVDR), are illustrated. Modified from Miyamoto *et al.* (1997). Copyright 1997, The Endocrine Society.

### 2.3.5.2 *VDR* associated disorders

The disorders of vitamin D metabolism are inherited metabolic abnormalities involving mutations of the vitamin D receptor, or of enzymes involved in the metabolism of vitamin D to its biologically active form, 1,25-dihydroxyvitamin D.

Familial target tissue insensitivity to 1,25(OH)<sub>2</sub>D<sub>3</sub> is known as hereditary hypocalcemic vitamin D-resistant rickets type II (VDRR). It is an autosomal recessive disorder characterized by the clinical, radiological and histological symptoms typical of hypocalcemic rickets and osteomalacia. In addition, VDRR patients have alopecia. VDRR type II is caused by nonsense, frameshift, or missense mutations in the gene encoding VDR. These mutations result in truncated VDRs, a damaged DNA-binding domain, a damaged hormone-binding domain, and/or impaired hetero-dimerization with the RXRs. (Hughes *et al.* 1988, Whitfield *et al.* 1996, Malloy *et al.* 1997, Haussler *et al.* 1998).

*VDR* is one of the genes that has been extensively studied in relation to osteoporosis. It is responsible for a broad range of actions of 1,25-(OH)<sub>2</sub> vitamin D, including its effect on calcium transport, homeostasis and bone resorption. Vitamin D interacts with its receptor and affects calcium homeostasis by regulating bone cell growth and

differentiation, calcium absorption and PTH secretion (Ralston 2002). The most widely studied *VDR* polymorphisms are *FokI*, *BsmI*, *ApaI* and *TaqI*. The *FokI* polymorphism creates an alternative translational start site (ATG) three codons from the downstream start site in exon 2 (Arai *et al.* 1997). The polymorphic restriction sites for the endonucleases *BsmI* and *ApaI* are located in the intronic sequence between exons 8 and 9. The fourth polymorphic site, for endonuclease *TaqI*, is situated in exon 9 (codon 352) and does not change the encoded amino acid, isoleucine. Morrison and coworkers (1994) showed that the three common allelic variants, *BsmI*, *ApaI* and *TaqI*, can be used to predict differences in bone density, accounting for up to 75% of the total genetic effect on bone density in healthy persons. However, studies of *VDR* in relation to bone mass have yielded inconsistent and conflicting results (Hustmyer *et al.* 1994, Lim *et al.* 1995, Houston *et al.* 1996). Studies of the *FokI* polymorphism in relation to BMD and fractures have also yielded conflicting results (Eccleshall *et al.* 1998, Ferrari *et al.* 1998). Thus, although, several polymorphisms have been associated with BMD, the mechanisms by which the *VDR* alleles regulate BMD are still poorly understood.

Even though a *VDR* polymorphism has been reported to be a determinant of bone formation and intestinal calcium absorption, there is no relationship between the polymorphism and osteomalacia (Kahraman *et al.* 2004).

### **2.3.6 Genetic factors in stress fractures**

The exact cause of stress fractures is not known. Predisposing environmental factors have been studied, but the genetic basis of stress fractures remains a relatively unexplored field. However, several observations suggest that a genetic component for stress fractures does exist. Firstly, Singer and coworkers (1990) described monozygotic twins with multiple stress fractures in identical anatomical sites. Secondly, multiple lower limb stress fractures have been reported in the same individuals. This suggests that the bone composition is defective and, thus, genetic factors are likely to play a role as predisposing factors. (Milgrom *et al.* 1985, Nielens *et al.* 1994, Lambros & Alder 1997).

Candidate genes for stress fractures are logically involved in bone formation, remodelling, or bone matrix formation, as are candidate genes involved in osteoporosis (Friedman *et al.* 2001). However, no candidate genes have yet been assessed with regards to a possible role in stress fractures.

## **2.4 Animal models of bone disorders**

Genetically engineered animal models of human bone disorders are extremely important for understanding the molecular mechanisms underlying the corresponding diseases and, by providing a more uniform experimental material, for investigating and testing possible therapies.

### 2.4.1 *Osteogenesis imperfecta*

Several approaches have been used for studying OI in mice. Two transgenic mouse models have been generated: one with a glycine substitution in *Colla1* (Stacey *et al.* 1988), and the other with an in-frame deletion in *Colla1* (Khillan *et al.* 1991). The first mouse model, with a glycine to cysteine substitution (G859C), mimics the most common OI mutations in humans. This mouse model exhibited a dominant lethal phenotype characteristic of the human disease, and allowed to demonstrate that as little as 10 % mutant gene expression can disrupt normal collagen function. The second mouse model, with a deletion of the 41 central exons coding for the collagen helix, developed a lethal phenotype when expressing high levels of *Colla1*. In contrast, the mice expressing lower levels of the mutant allele developed a milder phenotype, agreeing well with a dominant negative model. Since the mice were transgenic, the mutant gene is present in the genome in varying copy numbers and, therefore, the models give limited insight into OI pathophysiology and the development of gene therapy. (Stacey *et al.* 1988, Khillan *et al.* 1991.)

There are two mouse models exhibiting recessive transmission of a type I collagen defect. Firstly, Chipman and coworkers described an osteogenesis imperfecta-murine (oim) strain with a defect in the  $\alpha 2(I)$  C-terminal propeptide that prevented the incorporation of the  $\alpha 2$  chain into the procollagen (Chipman *et al.* 1993). This leads to the production of  $\alpha 1(I)$  homotrimers. The homozygous oim mouse had phenotypic features that simulated the human autosomal recessive OI type III. The production of only  $\alpha 1(I)$  homotrimers has also been described in one proband with a homozygous frameshift mutation in the *COL1A2* gene (Nicholls *et al.* 1983, Pihlajaniemi *et al.* 1984). Schnieke and coworkers created another strain of mice in which an insertion of retrovirus into the pro $\alpha 1(I)$  collagen gene resulted in a homozygous null *Colla1* gene, and in lethal embryo (Schnieke *et al.* 1983).

The first knock-in murine model for OI was generated with a heterozygous glycine substitution mutation (G349C) in the pro $\alpha 1(I)$  chain (Forlino *et al.* 1999). The phenotype of the mice varied from moderate to lethal. The results demonstrated that mice with a variable phenotype have an equivalent expression of mutant  $\alpha 1(I)$  mRNA in several tissues, and that discrete non-collagenous modifying factors are likely to be responsible for the variable severity in OI. (Forlino *et al.* 1999).

### 2.4.2 *Osteoporosis*

Complementary studies in animals are vital in searching for the susceptibility genes for osteoporosis. Animal models will continue to be important tools in the mission to understand the contribution of specific genes to peak bone mass and optimal bone architecture, and the genetic basis for a predisposition towards accelerated bone loss, as well as the potential therapeutic insights. Once a candidate gene has been identified, functional tests can be conducted in genetically altered mice by gene knockouts, or via the constitutive, or conditional expression of transgenes (Shmookler Reis & Ebert 2003).

The most commonly used animal to study osteoporosis is the mouse. A common approach is to identify chromosomal regions contributing to osteoporosis, or BMD, by

mapping quantitative traits in experimental animal models, and then to search syntenic regions of the human genome for genes defining these traits in humans. Then, using knockout and transgenic mice, the effects of the selected gene product on bone biology, as well as candidate genes for human studies, can be identified (Peacock *et al.* 2002). A number of knockout mice with an osteoporotic phenotype have been produced. For example, it has been showed that mice with a targeted disruption of *Lrp5* develop a low bone mass phenotype (Kato *et al.* 2002), mice lacking the vitamin D receptor exhibit an impaired bone formation (Yoshizawa *et al.* 1997), and osteoprotegerin-deficient mice develop early-onset osteoporosis (Bucay *et al.* 1998).

### ***2.4.3 Stress fractures***

The difficulty in identifying a suitable experimental animal model has limited the study of the pathophysiology of stress fractures. So far, only two rabbit models have been used in such studies. Using these models, it was shown that the micro-damage and the remodelling reaction are part of the process that underlies the pathogenesis of fracture development, agreeing well with the results obtained from the studies of human stress fractures (Li *et al.* 1985, Burr *et al.* 1990, Burr 2001). No animal models have been used to study the contribution of specific genes to the development of stress fractures.

### **3 Outlines of the present research**

The object of this work was to increase our knowledge-base on the role of genetic factors involved in bone disorders. The specific aims of the present study were:

1. to analyze the correlation between the genetic findings, clinical characteristics and hearing loss in patients with OI;
2. to analyze the correlation between genetic factors and bone mineral density in patients with juvenile osteoporosis using a candidate gene approach; and
3. to analyze the roles of selected candidate genes in the development of stress fractures and to estimate the significance of genetic factors in the pathogenesis of stress fractures.

## **4 Materials and methods**

### **4.1 Patients (I-III)**

A nationwide search was performed through the patient register of the Department of Clinical Genetics, Helsinki University Central Hospital, the membership register of the Finnish Osteogenesis Imperfecta Association, and the patient-care register of the Finnish University and Central Hospitals (HILMO) since 1998. 54 unrelated Finnish patients with a diagnosis of osteogenesis imperfecta, and fulfilling the inclusion criteria, were available for the study. The inclusion criteria of the study subjects were: diagnosis of OI based on multiple skeletal fractures due to inappropriately mild traumas, occurring particularly during childhood, a previous audiometric study and either a previously diagnosed hearing loss, or an age of greater than 35 years. A clinical geneticist established the diagnosis and classification of OI, on the basis of the fracture history, clinical and radiographic findings, and the family history.

Altogether, 20 patients with juvenile osteoporosis and their family members were referred to us from the pediatric osteoporosis clinic of the Hospital for Sick Children, Toronto. Patients with a known diagnosis, or clinical features suggestive of OI, or of OPPG (impaired vision, ligament laxity, mental retardation, or seizure disorder), as well as patients with an underlying chronic illness, or systemic medication, were excluded from the study. Experienced clinicians evaluated the patients clinically and radiologically. The diagnosis of osteoporosis was based on i) low BMD, defined as a z-score (standard deviation score) below  $-2.0$ , and/or ii) a history of increased bone fragility, defined as  $\geq 3$  peripheral fractures caused by low impact trauma, and/or iii) compression fracture(s). The group of 123 controls consisted of 88 patients with an established diagnosis of skeletal dysplasia (Schmid type of metaphyseal dysplasia, multiple epiphyseal dysplasia, or Ehlers-Danlos syndrome) and 35 healthy adults from the same geographical area as the patients. In each case, an experienced clinician had clinically and/or radiographically confirmed the diagnosis of skeletal dysplasia in the first control group; none of the controls presented with osteoporosis.

Military conscripts with a stress fracture of the femoral neck were referred to us from the military hospitals belonging to the Finnish Defence Forces. Altogether, 72 subjects were available for the study. The diagnosis of stress fracture had been based on accepted radiographic, scintigraphic, or MRI criteria, and these were checked and confirmed at the

follow-up examination. The control group consisted of 120 Finnish conscripts, for whom an experienced clinician ensured, from the military medical records and by means of a questionnaire, that none had sustained any stress fractures, either during military service, or before. Blood samples were collected from the 72 subjects with a femoral neck stress fracture and from the 120 controls, and genomic DNA was extracted from the blood by standard protocols.

## 4.2 CSGE (I-III)

To screen several human genes for mutations by conformation-sensitive gel electrophoresis (CSGE), the PCR products were amplified from genomic DNA. The PCR products were 200-600 bp in size and contained at least 60 bp of exon-flanking sequences (Ganguly *et al.* 1993, K rkk  *et al.* 1998b). PCR amplifications were carried out in a reaction volume of 23  $\mu$ l containing 40 ng of genomic DNA, 200  $\mu$ M of each dNTP, 0.25  $\mu$ M of each primer, and 1 unit of *TaqI* polymerase (AmpliTaq Gold, Applied Biosystems). The PCR conditions included an initial denaturation at 95°C for 10 min, followed by 34 cycles of 95°C for 40 sec, 54-64°C for 40 sec, and 72°C for 40 sec, followed by a final extension at 72°C for 10 min. To generate heteroduplexes for CSGE analysis, the PCR products were denatured at 98°C for 3 min and reannealed at 68°C for 30 min. The PCR products were analyzed by agarose gel electrophoresis to check the quantity and quality of the products. Before loading the PCR products onto a CSGE gel, the products were mixed with 10 x loading buffer (30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol FF).

A 1-mm thick gel was used for CSGE analysis in a standard DNA sequencing apparatus with 37.5 x 45 cm glass plates. The gel was prepared with 15% BAP-acrylamide (a 1:99 ratio of 1,4-bis(acryloyl)piperazine to acrylamide (Fluka, Intermountain Scientific)), 10% ethylene glycol (Sigma), 15% formamide (Gibco), 0.1% ammonium persulphate (APS; U.S. Biochemicals) and 0.07% N,N,N',N'-tetramethylethylenediamine (TEMED; Sigma) in 0.5 x TTE buffer (44 mM Tris – 14.5 mM taurine – 0.1 mM EDTA, pH 9.0) (Ganguly *et al.* 1993). The electrode buffer was 0.5 x TTE. The gel was pre-electrophoresed at 40 W for 20 min, and the samples were electrophoresed at 20 W for 10 hours at room temperature. After the electrophoresis, the gel was stained with SYBR Gold nucleic acid gel stain (Eugene). A hand-held UV torch was used to visualize the bands, and the relevant gel piece was cut out, and photographed with a high quality charge-coupled device (CCD) camera (Fotodyne, or UVP).

## 4.3 Screening of the *COL1A1*, *COL1A2* and *LRP5* genes for mutations (I, II)

Genomic DNA was isolated by standard procedures from the blood samples of 54 patients with OI, 20 patients with juvenile osteoporosis and 123 controls. Screening of the *COL1A1* and *COL1A2* genes was performed for the patients with OI. In addition to these two genes, the *LRP5* gene was analyzed for the patients with juvenile osteoporosis

and for the controls. PCR amplifications of the 51 exons of *COL1A1*, the 52 exons of *COL1A2*, and the 23 exons of *LRP5*, were performed as described previously (Gong *et al.* 2001, Körkkö *et al.* 1998a). Scanning of the PCR products was performed by CSGE, as described above in the Methods section 4.2. Following amplification, the quantity and quality of the PCR products were checked on an agarose gel. The CSGE analysis was then performed. The PCR products containing heteroduplexes in CSGE were sequenced using PCR primers and an automated instrument (ABI PRISM 377, or 3100 Sequencers and ABI PRISM Dye Terminator Cycle Sequencing Ready Kit, Applied Biosystems). Prior to sequencing, the samples were treated with exonuclease I to degrade the residual PCR primers, and shrimp alkaline phosphatase to dephosphorylate the residual nucleotides (Hanke & Wink 1994, Werle *et al.* 1994).

#### 4.4 RT-PCR (II)

RT-PCR was performed to study the effects of a frameshift mutation C913fs (c.2737\_2738insT) in exon 12 of a patient (number 16) with juvenile osteoporosis. Total RNA was extracted from Epstein-Barr virus-transformed lymphoblasts of patient 16 and of a control individual, using the RNeasy kit (Qiagen). The cDNA synthesis was carried out with the Superscript First Strand Synthesis System for RT-PCR (Invitrogen). cDNA amplification by PCR was performed with a pair of primers corresponding to exons 8 and 14 of *LRP5* (5'-GCA TCG AGC GGG TGC ACA AGG and 5'-GCT CCG CGT TGA CGA CGA TG). The product was sequenced with a primer corresponding to exon 12 (5'-CTG GAC AGA CTG GAA TCT GC). To determine the presence of a synonymous single nucleotide polymorphism (SNP) (c.3297C>T, exon 15) in the cDNA as found in the genomic DNA of the *LRP5* gene, a RT-PCR product was amplified with primers corresponding to exons 13 and 15 (5'-CAG TCG GAT GAT CCC GGA C and 5'-CGC TTC AGG TCC GCG TCC AC). Sequencing of the RT-PCR product was performed with the former primer.

#### 4.5 Candidate gene analyses for patients with stress fractures (III)

Genomic DNA was isolated by standard procedures from the blood samples of 72 patients with stress fractures and 120 controls. PCR amplifications of the 51 exons of *COL1A1*, the 52 exons of *COL1A2*, and the 23 exons of *LRP5*, were performed as described previously (Gong *et al.* 2001, Körkkö *et al.* 1998a), using primers designed to amplify the 8 exons of *OPG* and the 5 exons of *ESRI* (III). The PCR products were scanned for sequence variations by CSGE, as described in the Methods section 4.2. PCR products containing heteroduplexes were directly sequenced using the PCR primers and an automated instrument (ABI PRISM 377, or 3100 Sequencer and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Kit, Applied Biosystems).

Three polymorphisms of the *VDR* gene were genotyped by PCR and restriction enzyme digestion, as described previously (Morrison *et al.* 1994, Riggs *et al.* 1995, Gross *et al.* 1996). Briefly, PCRs were carried out with genomic DNA, in a final reaction volume of 25 µl, followed by incubation of 5 µl of the product at specific temperature for

one hour with universal buffer and 10 U of restriction enzyme. *FokI* SNP was determined by amplifying the corresponding 265-bp PCR fragment from exon 2 with a primer pair (5'-AGC TGG CCC TGG CAC TGA CTC TGC TCT and 5'-ATG GAA ACA CCT TGC TTC TTC TCC CTC). The PCR products were digested with *FokI* (New England Biolabs) at 37°C for 2 hours, and then electrophoresed in a 2% agarose gel containing ethidium bromide. The C→T (*FokI*) polymorphism converts an ATG codon for the first methionine to an ACG codon for threonine. The presence of a *FokI* endonuclease site generates two fragments of 196 bp and 69 bp in length, and dictates that a 427-residue isoform of VDR, M1, is expressed (so-named because it contains an ATG translational start site corresponding to codon +1). When ATG is changed to ACG, the translation begins three residues downstream and a 424-residue isoform of the receptor is expressed (M4). The two other polymorphisms, G to A variation (*BsmI*) in intron 8 and T to C variation (*TaqI*) in exon 9, were also analyzed. The latter variation is a silent mutation (ATT/ATC) affecting Ile352. Following restriction endonuclease digestion of the amplified products for 1 hour at 37°C (*BsmI*), or 65°C (*TaqI*), the digested PCR products were electrophoresed in a 2% agarose gel containing ethidium bromide. The presence of the *BsmI* site generates two fragments of 700 and 200 bp. The presence of the *TaqI* site results in three fragments of 290, 245 and 205 bp.

A C to T polymorphism in the calcitonin receptor gene (*CTR*) converting a CCG codon for Pro463, to a CTG codon for Leu, and a G to C promotor polymorphism at position -174 of the interleukin 6 (*IL6*) gene, were also genotyped (Nakamura *et al.* 1997, Lei *et al.* 2003). To analyze the former polymorphism, PCR products of 228 bp were digested by *AluI* at 37°C for one hour. The presence of an *AluI* site results in two fragments of 120 and 108 bp. The second polymorphism was analyzed with *NlaIII* restriction enzyme. PCR products of 198 bp in length were digested by *NlaIII* at 37°C for one hour. The G allele results in two fragments of 167 and 31 bp, and the C allele results in three fragments of 122, 45 and 31 bp.

Three polymorphisms in both *COL1A1* and *COL1A2* were genotyped. One of them, a G to T variation located at the transcription factor Sp1 consensus site in the first intron of *COL1A1*, was analyzed directly by sequencing. One of the *COL1A1* polymorphisms, IVS45+31C>T, was analyzed with restriction enzyme *Eco57I*. The polymorphism of *COL1A2*, IVS28-41G>A, was analyzed with restriction enzyme *MspI*. The PCR products of three other polymorphisms, IVS28-14T>C in *COL1A1*, IVS6-68A>G, and IVS38-89ins38bp in *COL1A2*, were analyzed by CSGE, as described in the Methods section 4.2.

#### 4.6 Statistical analysis (I, III)

Analyses to evaluate statistical dependencies between the mutation types and the onset, presence, type, special characteristics and severity of hearing loss in patients with OI, were performed at the Department of Mathematics and Statistics of the University of Vaasa, Vaasa, Finland, utilizing t-tests and cross-tables (contingency tables). Due to the small sample size, in the cases of 2 by 2 cross-tabulations, we utilized the Fisher's exact test for testing the independencies instead of the traditional chi-square test. The advantage of the Fisher's exact test is that it does not rely on the large sample theory, as does the chi-square analysis, and should therefore be more powerful in detecting potential dependencies.

The statistical analyses in the case-control association study concerning stress fractures were performed at the Laboratory of Statistical Genetics of Rockefeller University, New York, USA. For each SNP, genotypes in control individuals were tested for deviations from the Hardy-Weinberg equilibrium. The analyses for disease association were carried out on alleles and genotypes by means of likelihood ratio chi-square tests, as no significant deviations were found. For each gene with more than one SNP, haplotype frequencies were estimated separately for case and control individuals. The haplotype frequencies were estimated by maximum likelihood (the *snphap* program), and likelihood ratio tests were carried out to evaluate the differences in haplotype frequencies between the cases and controls (Chiano & Clayton 1998). To investigate the effects of combined genotypes at multiple SNPs, haplotypes were inferred to individuals with the *snphap* program, and all possible genotypes (pairs of haplotypes) were evaluated. The *snphap* program is available at <http://www-gene.cimr.cam.ac.uk/clayton/software>.

## 5 Results

### 5.1 Genotype-phenotype comparison of patients with osteogenesis imperfecta (I)

A total of 54 Finnish OI patients with previously diagnosed hearing loss, or aged older than 35 years, were analyzed for mutations in the *COL1A1*, or *COL1A2* genes by CSGE. Altogether, 49 mutations were identified, of which 41 were novel. Thirty-two of the 49 patients had familial OI and 16 had sporadic disease. One patient could be classified as neither familial, nor sporadic, since the family members could not be examined.

#### 5.1.1 Identification of mutations in the *COL1A1* and *COL1A2* genes in patients with osteogenesis imperfecta

Patients with OI were screened for mutations in the *COL1A1* and *COL1A2* genes. The 51 exons of *COL1A1*, the 52 exons of *COL1A2*, and the exon boundaries of the genes, were amplified by PCR, denatured, and annealed to generate the heteroduplexes essential for CSGE analysis. The products were analyzed on agarose gels to check their quantity and quality, followed by CSGE. Several heteroduplexes were observed. Sequencing identified 49 mutations in *COL1A1* and *COL1A2*, of which 38 mutations were in *COL1A1* and 11 were in *COL1A2*. In five patients, the CSGE analysis and complete sequencing of the genes did not detect any mutations. Two pairs of unrelated patients presented an identical mutation. Sixteen patients had a single-base mutation that converted codons for obligate glycines in the triple helix of type I collagen to codons for bulkier amino acids. Six glycine mutations were situated between exons 13 and 45 in the *COL1A1* gene, while the other ten were located between exons 19 and 49 in the *COL1A2* gene. No correlation was observed between the locations of the glycine substitutions and the severity of the OI (Table 1 in paper I).

Sixteen RNA splicing mutations were detected. Eleven of these altered consensus sites of RNA splicing in the conventional AG-dinucleotide at the acceptor splice site, or the GT-dinucleotide at the donor site. The mutations were located between introns 1 and 44

of the *COL1A1* gene, and one was situated in intron 43 of the *COL1A2* gene. The five remaining mutations were also likely to alter RNA splicing. One patient with OI type III had a two nucleotide deletion in exon 33/34 of *COL1A1* (c.2268\_2269delTC). Since this patient had severe OI, the mutation was predicted to result in nonsense-mediated exon skipping instead of haploinsufficiency, and was therefore included in this group. Unfortunately, RNA was not available to substantiate this prediction. In addition, one patient with OI type I had a 4-bp deletion, from position +3 to position +6, in intron 17 (c.1155+3\_1155+6delAAGT). Of the three remaining mutations, one was at position -3 in intron 1, the second at position +3 in intron 40, and the third at position +5 in intron 44. Although the base at the position -3 is not conserved in all introns, this sequence variant has not been reported earlier, and was not observed in the other 53 patients with OI. Previously, two mutations at position -3 in the *COL1A1* gene, one in intron 43 and another in intron 47, have been reported in patients with OI type II and type III/IV (Byers 1990, Pepin *et al.* 1997).

Thirteen frameshift and four nonsense mutations were found in the *COL1A1* gene. These mutations predict premature termination of translation and cause a dramatic reduction in the amount of transcript from the mutant allele (nonsense-mediated mRNA decay). Deletions of one, or two nucleotides in the coding sequence resulted in frameshift mutations in nine patients. Single nucleotide insertions also caused frameshift mutations in four patients. One frameshift mutation (c.299\_300insC) occurred in two unrelated patients. Of the four nonsense mutations, Arg1026X occurred in two unrelated patients with OI type I.

### ***5.1.2 Hearing loss in patients with osteogenesis imperfecta***

Of the 49 patients with a mutation in either *COL1A1*, or *COL1A2*, 32 had hearing loss (65.3 %) (Table 2 in paper I). Hearing loss was bilateral in 24 patients and unilateral in 8 patients. The mean age at the onset of hearing loss was 23.9 years (SD 8.0, range 12-45 years). Hearing loss was found in all OI types.

According to the audiometric studies, 15 of the 32 patients with hearing loss had early sensorineural hearing loss. In this type of hearing loss, bone conduction thresholds for the frequencies between 500-2000 Hz were 20 dB, or more, with corresponding air-bone gaps smaller than 15 dB before the age of thirty years. One patient had anacusis. Five patients had bilateral conductive hearing loss, in which the average air-bone gap for the frequencies 0.5, 1 and 2 kHz were greater than 15 dB, with corresponding bone conduction thresholds smaller than 15 dB. Six patients had bilateral mixed hearing loss, in which the average air-bone gap for the frequencies 0.5, 1 and 2 kHz were greater than 15 dB, with corresponding bone conduction thresholds equal to, or greater, than 15 dB. The inheritance pattern of hearing loss could be studied in 14 cases.

### ***5.1.3 Genotype-phenotype comparison***

The OI patients with *COL1A1* and *COL1A2* mutations were classified in two ways. In the first classification, mutations were divided into those that were situated in *COL1A1* and

those in *COLIA2*. The presence of different clinical phenotypes characteristic of OI was compared in patients with *COLIA1* and *COLIA2* mutations. The compared clinical characteristics included the inheritance pattern, OI type, height, colour of sclerae, fracture rate and hearing status. Dentinogenesis imperfecta was not evaluated, since radiological, or histological examinations of the patients' teeth were not available.

There were some differences in the patients' phenotypes. The patients with *COLIA1* mutations more frequently had OI types I and III. In contrast, patients with *COLIA2* mutations had a clinical phenotype of OI type IV ( $p = 0.00086$ , t-test). Furthermore, blue sclerae were observed more often in the patients with *COLIA1* mutations. Slight differences were also observed in height. The patients with *COLIA2* mutations tended to be shorter than those with *COLIA1* mutations ( $p = 0.0443$ ). Hearing loss seemed to be equally common in the cases with *COLIA1* and *COLIA2* mutations.

In the second classification, the mutations were divided into those that are predicted to alter the type I collagen structure and those that destabilize the mRNA and result in haploinsufficiency. In the first group, the mutations resulting in glycine substitutions in pro $\alpha$ 1(I), or pro $\alpha$ 2(I), and mutations that altered RNA splicing were predicted to alter the structure of the type I collagen. In the second group, the mutations that were expected to result in haploinsufficiency included nonsense and frameshift mutations, which typically lead to NMD of mRNA through the creation of premature termination codon. The association between the two different mutation types and clinical OI types were statistically significant ( $p = 0.0088$ ). This was predominantly due to the appearance of haploinsufficiency mutations only in patients with OI type I (Table 3 in paper I). Furthermore, the cases with the most severe type of OI had only structural mutations. The other clinical findings, including the inheritance pattern, fracture rates, hearing status, blue sclerae and height, did not differ between the two groups.

## 5.2 Detection of mutations in the *LRP5* gene in patients with primary osteoporosis (II)

Twenty patients fulfilling the criteria for juvenile osteoporosis, who lacked phenotypic features of OI and OPPG, were analyzed by CSGE for mutations in the *COLIA1*, *COLIA2* and *LRP5* genes. Altogether, three mutations were identified in the *LRP5* gene. No mutations were found in the *COLIA1* and *COLIA2* genes.

### 5.2.1 Identification of mutations in the *LRP5* gene by CSGE

Mutation analyses of all *COLIA1* and *COLIA2* exons and exon boundaries were performed by CSGE and sequencing. No putatively disease-associated mutations were found. We then continued to analyze the *LRP5* gene for mutations, screening the 23 exons and exon boundaries of the *LRP5* gene in the 20 patients. CSGE and subsequent sequencing identified three novel heterozygous mutations and eight SNPs. Two of the mutations were missense mutations: A29T (c.85G>A) and R1036Q (c.3107G>A) in exons 1 and 14, respectively, and one was a frameshift mutation C913fs (c.2737\_2738insT) in exon 12, which predictably results in a premature termination

codon in exon 13. Similar sequence variations were not observed in any of the 123 controls, nor in the patients' healthy family members. Four of the eight detected SNPs were synonymous (E644E, D1099D, V1119V and P1241P). One of the synonymous SNPs was novel (P1241P) and was not found in the controls, but it is unlikely to affect the protein function. Seven of the SNPs were also found in the control samples.

RT-PCR analysis was performed to study the consequences of the C913fs mutation. Total RNA was isolated from EBV-transformed lymphoblasts of the patient and a control, and used as a template for cDNA synthesis. One PCR product of 1534 bp was obtained from both samples, but sequencing of the patient's RT-PCR product indicated only the presence of the normal allele. As a next step, both the cDNA and genomic DNA of the patient were analyzed for the presence of a synonymous SNP, c.3297C>T (D1099D), in exon 15. CSGE analysis and sequencing of the genomic DNA showed that the patient was heterozygous for the SNP, but sequence analysis of the cDNA revealed the presence of only one allele. Thus, the presence of only the wild-type cDNA sequence indicates that the frameshift mutation in the *LRP5* gene resulted in haploinsufficiency through nonsense-mediated mRNA decay of the mutated allele.

## 5.2.2 Genetic and clinical findings

Twenty patients (11 males, 9 females) with juvenile osteoporosis, with a mean age of 10.5 years (range 4.0-16.0 yrs) at the time of the study, were clinically evaluated before candidate gene analyses. Eighteen of the 20 patients had had one, or more peripheral fractures, and 15 patients had had  $\geq 3$  peripheral fractures. In three patients, the total number of peripheral fractures exceeded eight and, in the others, ranged from 1 to 6 fractures. Spinal radiographs were available for 15 patients. Seven patients had multiple compression fractures and five had other significant osteoporotic changes in the vertebral body morphology. Only 5 patients had normal spinal radiographs. The spinal BMD mean z-score of the 18 patients who were  $\geq 5$  years old was  $-2.1$ ; it was below  $-2.0$  in nine patients and above  $-1.5$  in five patients. The 20 patients did not have any symptoms suggestive of an underlying chronic illness, or significant hypocalcemia, hypophosphatemia, hyperparathyroidism, or hypercalciuria, which could result in the weakening of the bones. All had normal 25-OH-vitamin D serum concentrations. The mean serum alkaline phosphatase (ALP) concentration was  $-1.7$  SD. Impaired vision, or other significant ophthalmological problems typical of OPPG, were not reported in any of the patients.

All three *LRP5* mutations were found in the coding region of the *LRP5* gene. Two of them were missense mutations: A29T (c.85G>A) and R1036Q (c.3107G>A) in exons 1 and 14, respectively, and one was a frameshift mutation C913fs (c.2737\_2738insT) in exon 12. Neither the 123 controls, nor the patients' healthy family members, had the same mutations as the patients. The three *LRP5* gene mutations have not been reported earlier.

At the age of 16 years, the patient with the missense mutation A29T had a history of four peripheral fractures (at ages 4, 7, 8 and 14 years). Her height was 165 cm, the BMD z-score was  $-1.9$  for the lumbar spine, and spinal radiographs showed multiple compression fractures of the vertebral bodies. Her father had a history of one sport-related fracture and her mother had had no fractures. The parents had normal BMDs, had

no evidence of osteoporosis, and neither carried the same sequence change in the *LRP5* gene as the proband.

The patient with the missense mutation R1036Q had a history of five peripheral fractures between the ages of 14 months and 5 years; all fractures were associated with low impact trauma. His height at the age of 9 years was 144 cm and the BMD z-score was -0.1. The proband's mother had a history of three peripheral fractures resulting from low to moderate impact traumas, and was found to have the same mutation as the proband. In contrast, the father's fracture history was negative and he did not carry the mutation.

The third mutation was a frameshift mutation C913fs (c.2737\_2738insT) in exon 12, which was predicted to result in a premature termination codon in exon 13. The patient had had a humerus fracture at the age of 2 years. At the age of 16 years, he was found to have a compression fracture after a sport-related injury. He was a healthy post-pubertal adolescent with a normal height (175.6 cm), no limb deformities and normal vision. His BMD was markedly reduced (z-score -2.9) and the evaluation of the spinal radiographs showed an osteopenic appearance and compression deformities of variable severity in the vertebrae. His 18-year-old brother was also found to have a significantly reduced BMD (z-score -3.3) and spinal changes, but no peripheral fractures. Based on these findings, the parents were also assessed for osteoporosis. The proband's father, age 54, was asymptomatic and had a negative fracture history. His BMD T-score was -1.9, and he had multiple compression fractures. Both the father and the brother were found to have the same mutation as the proband. The mother had normal a BMD and did not carry the frameshift mutation.

### **5.3 Identification of a haplotype of the *VDR* gene associated with stress fractures (III)**

#### ***5.3.1 Candidate gene analysis in stress fractures***

A total of 72 subjects with femoral neck stress fractures were analyzed for mutations in the *COL1A1*, *COL1A2*, *LRP5*, *OPG* and *ESR1* genes by CSGE and sequencing. All 51 exons of *COL1A1*, the 52 exons of *COL1A2*, the 23 exons of *LRP5*, the eight exons of *OPG*, the five exons of *ESR1*, and the exon boundaries, were screened for mutations. The analysis did not reveal any putative disease-causing mutations. Several sequence variations were found in these genes, but none of them was unique, as they were detected in both stress fracture subjects and controls.

#### ***5.3.2 Allelic association analysis***

To test for possible allelic associations, we initially studied 11 polymorphisms in the *COL1A1*, *COL1A2*, *CTR*, *IL6* and *VDR* genes. The 11 SNPs were genotyped in 72 cases and 120 controls. The resulting allele frequencies were compared between the cases and controls (Table 2 in paper III). Only one of the polymorphisms, *TaqI* (C to T variation) in

*VDR*, showed a suggestive association with stress fractures ( $p = 0.033$ ), but this association could not be considered significant when multiple testing was taken into account. The allele frequencies of the other 10 SNPs did not differ significantly between the two groups.

### 5.3.3 Haplotype analysis

Although the genotyping results of the two-allelic polymorphisms provided no strong evidence for an association, the finding of a suggestive allelic association between the *VDR* polymorphism and the phenotype prompted us to perform a haplotype analysis.

The *VDR* haplotypes were constructed from the *FokI*, *BsmI* and *TaqI* restriction site polymorphisms. The analysis revealed highly significant overall differences in haplotype frequencies between the patients and controls for *VDR* haplotypes ( $p = 0.000012$ ). This was mainly due to the association between the phenotype and the two *VDR* haplotypes, TGT (OR = 1.71, 95% CI = 1.05-2.96) and CGT (OR = 1.71, 95% CI = 1.09-2.62) (Table 3 in paper III). The TGT haplotype frequencies were 0.24 for the cases and 0.16 for the controls. As the differences were not significant between cases and controls, we constructed pairs of haplotypes for each haplotype. All possible genotypes (pairs of haplotypes) were evaluated, although not all the possible pairs were observed (Table 4 in paper III). The TGT/TGT homozygote was the genotype most closely associated with the phenotype. The individuals who were homozygous for the TGT haplotype had a five-fold higher risk of stress fractures (OR = 5.23, 95% CI = 1.60 – 17.11), with an associated attributable risk of 12% (95% CI = 3% – 21%). That is, 12% of the individuals with femoral neck stress fractures in the population are estimated to have this condition as a consequence of being homozygous for the TGT haplotype. In other words, 88 % of patients with stress fractures are affected for reasons unrelated to this particular haplotype in the *VDR* gene.

## 6 Discussion

### 6.1 Methodological considerations

There are a number of factors that affect the mutation detection frequency, one of the most important of which is the clinical diagnosis. A clinical geneticist established the diagnosis and classification of OI, based on the fracture history, clinical and radiographic findings, and the family history. The 32 OI patients had 74 relatives with diagnosed OI. Therefore, our 49 patients represent the molecular genetic background of 123 Finnish OI patients. The clinical criteria of JIO was based on i) a low BMD, defined as a z-score (standard deviation score) below  $-2.0$ , and/or ii) a history of increased bone fragility, defined as  $\geq 3$  peripheral fractures caused by low impact traumas, and/or iii) compression fracture(s). Since a low BMD does not reveal the quality of bone, it was not used as a single criterion as in adult osteoporosis. It can be difficult to establish a definite osteoporosis diagnosis in children and, therefore, molecular diagnosis could benefit these patients. The diagnosis of stress fracture was based on widely-accepted radiographic, scintigraphic, or MRI criteria (Anderson & Greenspan 1996, Kiuru *et al.* 2004).

The second important factor is the sensitivity of the screening method. It is difficult to assess the absolute degree of specificity, or sensitivity, of any single method. However, the screening of the fibrillin 1 gene for mutations by CSGE has been shown to detect at least 90 % of the known mutations (Körkkö *et al.* 2002). The finding of the high sensitivity and specificity of CSGE is also supported in a study in which all known sequence variations of six collagen genes, *COL1A1*, *COL1A2*, *COL2A1*, *COL3A1*, *COL9A1* and *COL9A2*, were detected using this method (Körkkö *et al.* 1998b).

The third factor is the nature and location of the mutation, which can also affect the detection rate. It has been shown that CSGE is more sensitive to mismatches in an AT-rich sequence context than in a GC-rich context (Ganguly 2002). CSGE also has a limited resolution of two closely linked sequence variations (Ganguly 2002). Furthermore, CSGE will not detect large, multi-exon rearrangements. This may partially explain why no mutations were found in five of the 54 OI patients. Nevertheless, the CSGE method is one of the most powerful and cost-efficient tools, with high sensitivity and specificity, to scan genes for mutations.

## 6.2 Association between OI phenotypes and genotypes

OI is a genetic disorder with a wide range of clinical severity, varying from perinatal lethality, to very mild forms without fractures (Byers & Cole 2002). It has been assumed that the resulting phenotype depends on which of the two  $\alpha$  chains is affected, the position in the triple helix at which the mutation arises, and which amino acid substitutes for glycine (Rauch & Glorieux 2004). To study the genotype-phenotype correlation, the *COL1A1* and *COL1A2* genes in 54 patients with OI were screened for mutations by CSGE.

A total of 49 mutations were detected, 38 in the *COL1A1* gene and eleven in the *COL1A2* gene. Forty-one of the mutations were novel. The 49 mutations consisted of sixteen single-base mutations resulting in glycine substitutions, sixteen splicing mutations, four nonsense mutations and thirteen frameshift mutations. The different phenotype characteristics of OI patients with either structural mutations, or mutations that result in the reduction of the amount of type I collagen, were compared together. The association between structural and haploinsufficiency mutations and clinical OI types were statistically significant, mainly because the haploinsufficiency mutations were present only in patients with OI type I (Table 3 in paper I). The finding that haploinsufficiency mutations result in a mild OI type has also been observed in several other studies (Willing *et al.* 1992, 1994, 1996, Körkkö *et al.* 1998a). The transcription products of the genes with haploinsufficiency mutations are usually unstable and are destroyed by NMD of mRNA. As a result, the amount of normal type I collagen is reduced, leading to the mild phenotype (Byers 2000, Rauch & Glorieux 2004). When other clinical features, such as blue sclerae, height and fracture rate, were compared with the mutation types, no correlations were found.

The *COL1A1* mutations were present more frequently in patients with OI types I and III, and *COL1A2* mutations were more frequent in OI type IV ( $p = 0.00086$ ). The patients with *COL1A1* mutations had blue sclerae more often than the patients with *COL1A2* mutations. Furthermore, patients with *COL1A2* mutations tended to be shorter than those with *COL1A1* mutations ( $p = 0.0443$ ). These results were not surprising, because structural mutations are typically seen in the moderate to severe forms of the disorder, and haploinsufficiency mutations have not been observed in *COL1A2*.

*COL1A1* and *COL1A2* CSGE analysis and complete sequencing of the exons detected no mutations in five patients. The screening of the *COL1A1* and *COL1A2* genes for mutations by CSGE has been shown to detect at least 90 % of all type I collagen mutations (Körkkö *et al.* 1998b, Marlowe *et al.* 2002). In addition to this molecular genetic approach, biochemical testing is now also available to assist in the diagnosis of OI, and this approach also identifies 90 % of subjects known to have OI (Marlowe *et al.* 2002). All patients in our study had clinically confirmed OI. It is possible that these five patients had large gene rearrangements, which could not be detected by the mutation detection approach used here. Another possibility is that these patients demonstrate genetic heterogeneity and have mutations in genes other than the type I collagen genes. One of these patients had OI type V, which is characterized by moderate to severe bone fragility. While the heredity of this OI type V is autosomal dominant, no evidence for *COL1A1* and *COL1A2* gene mutations has been reported (Glorieux *et al.* 2000).

Progressive hearing loss is a common finding in OI. About half of patients older than 50 years of age report hearing loss, and a higher proportion of adults have pathological

audiometric findings (Paterson *et al.* 2001, Kuurila *et al.* 2002). However, very little is known about the possible correlation between a genotype and hearing loss phenotype in OI. It has been suggested that hearing loss is more common in OI type I than in type IV (Sillence 1981, 1988, Paterson *et al.* 2001). OI type I, with very little phenotypic variation, is generally caused by mutations that reduce the amount of type I collagen, but the mutations that alter the structure of type I collagen cause unpredictable phenotypes (Byers *et al.* 1991, Byers & Cole 2002). On the other hand, the auditory ossicles of the middle ear consist mainly of type I collagen (Birchall *et al.* 1982). In addition, the *COLIA2* gene has been isolated from a human fetal cochlear cDNA library, suggesting a role in the development of the cochlear structures of the inner ear (Robertson *et al.* 1994). Thus, it is possible that altered type I collagen fibrils could be responsible for sensorineural and conductive hearing loss. For these reasons, it was plausible to assume that certain mutations, particularly those resulting from *COLIA1* haploinsufficiency, or those occurring in *COLIA2*, may lead to a similar hearing loss phenotype.

In our study, no correlation was observed between the mutation types and the presence, type, or severity of hearing loss, the age of onset of hearing loss, or special features of the hearing loss, such as early sensorineural hearing loss and the expression of hearing loss in the family. On the contrary, distinct hearing phenotypes were observed in patients with identical mutations. Two patients with the same *COLIA1* haploinsufficiency mutation, c.299\_300insC, had different hearing patterns; one patient had normal hearing at the age of 56 years, while the other already had a moderate mixed/sensorineural hearing loss (right/left ear) at the age of 17 years. Furthermore, the Arg1026X mutation in the  $\alpha 1(I)$  chain resulted in a conductive hearing loss in one patient at the age of 39 years, and mixed hearing loss in another at the age of 50 years. Although, Sykes and coworkers (1990) suggested that hearing loss was more common in OI patients with *COLIA1* mutations than in those with mutations in *COLIA2*, the *COLIA1* mutations in our study were as likely to cause hearing loss as the *COLIA2* mutations.

Our results indicate that the pathogenesis of hearing loss is complex and cannot be explained by differences in the mutation types present in the type I collagen genes. Even though a mutation in type I collagen gene is essential, it is not sufficient to produce hearing loss. This is emphasised by the finding that hearing loss can be present only in some of the affected members of a family with OI. Apparently, hearing loss in OI is a consequence of multifactorial, still unidentified, genetic effects. The unpredictable occurrence of hearing loss may also be due to differences in gene expression, which may be modified by as yet undiscovered mechanisms. Thus, the variable expression of mutated *COLIA1*, or *COLIA2*, may result in an unpredictable fragility of the auditory ossicles' or cochlear hair cells' lamina. Variations of the hearing phenotype are also known in other conditions, such as Waardenburg syndrome (Lalwani *et al.* 1996). In addition, over 120 independent genes for deafness have been identified (Nance 2003). It is therefore likely that alterations in other genes involved in hearing loss act as genetic modifiers in the hearing phenotype of OI patients.

### **6.3 *LRP5* gene mutations and primary osteoporosis**

To study the role of genetic factors in primary osteoporosis, we performed a candidate gene study, first to determine whether the *COLIA1* and *COLIA2* genes have an effect on

the development of primary osteoporosis and, secondly, to exclude OI. A mutation in the *COL1A2* gene (G436R) had already been identified in two siblings with a history of back pain and with diagnosed juvenile osteoporosis (Dawson *et al.* 1999). However, of our twenty patients with a tendency for fractures and/or a low BMD, but without other features of OI, none had mutations in the *COL1A1* and *COL1A2* genes. We then analyzed the *LRP5* gene, which has recently been associated with different bone conditions, OPPG and high bone mass phenotypes (Gong *et al.* 2001, Little *et al.* 2002, Van Wesenbeeck *et al.* 2003, Ferrari *et al.* 2004). Three patients were found to have novel heterozygous mutations: A29T, C913fs and R1036Q, which affect the first, third and fourth YWTD/EGF domains of the LRP5 protein.

All *LRP5* mutations that have been identified in HBM phenotypes (Boyden *et al.* 2002, Little *et al.* 2002, Van Wesenbeeck *et al.* 2003) affect conserved amino acids of sequences encoded by exons 2, 3 and 4 of the *LRP5* gene, and which are all located in the first YWTD/EGF domain of the LRP5 protein (Figure 1 in paper II). The mechanism of action of the HBM-causing mutations in the *LRP5* gene is gain-of-function, resulting in increased Wnt signalling by impairing the action of a normal Wnt antagonist, dickkopf-1 (Boyden *et al.* 2002). In contrast to the HBM phenotypes, the mutations identified in OPPG are primarily located in the second and third YWTD/EGF domains, and their mechanism of action is loss-of-function (Gong *et al.* 2001). The finding that mutations in a single gene result in more than one phenotype is not uncommon. For example, loss-of-function mutations in the *PAX3* gene cause Waardenburg syndrome, and gain-of-function mutations lead to the development of a childhood tumor, alveolar rhabdomyosarcoma (Tassabehji *et al.* 1992, Barr *et al.* 1993). The observation that the location of the mutation in the *LRP5* gene might have an impact on the phenotype is also interesting. We can assume that the location of a mutation in the functionally different LRP5 domains impacts the LRP5 activity and/or Wnt signalling by different mechanisms, and thus results in either high, or low bone mass phenotypes.

In the present cohort, the mechanisms by which the mutations result in osteoporosis were not studied, but we can presume the following. The frameshift mutation in exon 12 is likely to result in haploinsufficiency through NMD of mRNA. The two missense mutations most likely alter the protein structure and result in a functionally abnormal protein, abolishing, or interfering with the binding between LRP5 and one of the Wnt proteins (Mao *et al.* 2001b, Patel & Karsenty 2002).

We measured the serum ALP from the 20 patients, and found that it was below the age- and sex-specific reference values in six patients (Table 1 in paper II). Two of the six patients had a mutation in the *LRP5* gene. This finding suggests that impaired Wnt/LRP5 signalling could result in low ALP levels. This is supported by a recent observation showing that the Wnt/LRP5 signalling cascade controls the expression of ALP (Rawadi *et al.* 2003). Furthermore, our results suggest that other proteins involved in the Wnt signalling pathway can also have a role in osteoblast activity and cause low bone mass in these patients. This, however, needs to be addressed in future studies.

In addition to the three mutations, eight SNPs were observed within the coding region of the *LRP5* gene in the 20 patients (Table 2 in paper II). Since four of the SNPs were synonymous (E644E, D1099D, V1119V and P1241P), they are unlikely to affect the protein function. Seven of the SNPs were also found in the control samples and, therefore, cannot be regarded as the cause of significant osteoporosis and fractures seen in the study subjects. However, it is possible that some of these SNPs may impact bone mass accrual and contribute to the variations of bone mass seen even in the general

population. This is supported by the recent findings indicating that a *LRP5* allelic variation contributes significantly to vertebral bone mass and size in men, by influencing vertebral bone growth during childhood (Ferrari *et al.* 2004).

It has been supposed that JIO is not a heritable disease, because of negative family history (Lorenz 2002). In our study, the family history of osteoporosis was evident in some of the patients, and the clinical phenotype showed similarities between affected family members. According to our results, JIO is a dominantly inherited bone disease and, at least in a subset of the families, is associated with mutations in the *LRP5* gene. A dominantly-inherited susceptibility, a positive family history for osteoporosis, and/or fractures, were also present in some of the seventeen patients who had no mutations in the *LRP5*, *COL1A1* and *COL1A2* genes.

Our results show, for the first time, that heterozygous mutations in the *LRP5* gene can result in primary juvenile osteoporosis characterized by low BMD, or/and increased bone fragility, without associated ocular manifestations. Our observations suggest that *LRP5* mutations may also play a role in the general adult population with osteoporosis. This is supported by the finding that the parents of the patients who were available for the study also had a low BMD, or/and an increased bone fragility. Whether *LRP5* mutations contribute to adult osteoporosis needs to be elucidated by future studies. The finding that *LRP5* mutations can cause JIO allows us to envisage the possibility of an improved diagnosis through genetic testing and counselling.

#### **6.4 *VDR* gene haplotype is associated with femoral neck stress fractures**

The etiology of stress fractures is likely to be multifactorial, with a well-defined environmental component. A role has been suggested for genetic factors, but no susceptibility genes have yet been identified (Friedman *et al.* 2001).

We generated haplotypes of three polymorphic sites, *FokI*, *BsmI* and *TaqI*. The analysis revealed highly significant differences in the haplotype frequencies between the patients and controls ( $p = 0.000012$ ), which were mostly due to the association between the phenotype and the two *VDR* haplotypes, TGT (OR = 1.71, 95% CI = 1.05-2.96) and CGT (OR = 1.71, 95% CI = 1.09-2.62). Furthermore, all possible pairs of haplotypes were evaluated. The TGT/TGT homozygote was the genotype most closely associated with the phenotype (OR = 5.23), suggesting that it acts in a recessive manner and only homozygotes are predisposed to stress fractures. We estimated that 12% of the affected individuals have the disease because they carry the TGT/TGT haplotype. Individuals carrying this homozygous haplotype have a five-fold higher risk of suffering a femoral neck stress fracture. However, our results do not demonstrate a direct causal role for the etiology of the stress fractures. It is possible that other variations in the *VDR* gene, or in the other genes in this locus, explain the observed association.

The TGT/TGT haplotype consisted of three polymorphic sites: *FokI*, *BsmI* and *TaqI*. It has been shown that the absence of the *FokI* endonuclease site dictates the expression of the M4 isoform, which is significantly more effective in activating transcription than the longer isoform receptor, M1 (Arai *et al.* 1997). It has been suggested that the M4 variant may provide partial protection against osteoporosis by ensuring enhanced bone mineral

density via the increased activity of VDR throughout life (Haussler *et al.* 1998). Our finding that the TGT/TGT genotype increases the risk of stress fractures is in accordance with these findings, because the TGT haplotype results in the production of the longer, less protective, M1 isoform. The two other polymorphic sites, *BsmI* and *TaqI* RFLPs, are located near the 3'-end of the gene, and are the most frequently used markers in *VDR* gene association studies. Morrison and coworkers (1994) were the first to report that the *BsmI* polymorphism was related to BMD in post-menopausal women. Since then, *BsmI* and *TaqI* RFLPs have been linked to several bone-specific parameters, such as BMD, fracture risk and bone turnover (Tao *et al.* 1998, Morrison *et al.* 1994, Langdahl *et al.* 2000, Braga *et al.* 2002, Laaksonen *et al.* 2002). However, these polymorphisms are not likely to be functional and, for this reason, the explanation for these findings is not known. These SNPs can still be used as markers, since they may be linked to another functional sequence variation elsewhere in the gene.

The results presented here showed, for the first time, that femoral neck stress fractures are associated with a certain *VDR* gene haplotype and genotype. 12% of the individuals with femoral neck stress fractures are predisposed to this condition as a consequence of being homozygous for the TGT haplotype. The remaining 88% of the cases are likely to be associated with other genetic and environmental factors, further demonstrating the multifactorial nature of the stress fractures. The role of genetic factors in stress fractures is indicated in this study, and is further supported by a twin study and other studies (Milgrom *et al.* 1985, Singer *et al.* 1990, Nielens *et al.* 1994, Lambros & Alder 1997). The detection of predisposing factors for stress fractures is likely to have an effect on their prevention. Prevention could be planned for individuals at high risk by decreasing their cyclic and intensive physical training by means of rest periods and increasing the observation of stress-related pain symptoms.

## 7 Conclusion

The roles of genetic factors associated with the *COL1A1*, *COL1A2*, *LRP5* and *VDR* genes in three different bone disorders, OI, JIO and stress fractures, were studied. The aim was to acquire new information concerning genotype-phenotype correlations, and to identify disease-causing, or predisposing genetic variations in these disorders.

To analyse the correlation between genetic findings, clinical characteristics and hearing loss in patients with OI, the *COL1A1* and *COL1A2* genes were screened for mutations in 54 patients. Altogether, 49 mutations were identified, of which 41 were novel. It was hypothesized that the mutated gene, or the mutation type, could lead to different types of hearing loss in OI. However, no correlation was observed between the mutated gene, or mutation type, and the hearing pattern. This suggests that the basis of hearing loss in OI is complex, and results from multifactorial genetic effects that remain to be identified. Analysis of the *LRP5* gene resulted in the identification of heterozygous missense, nonsense and frameshift mutations in three patients with juvenile osteoporosis. Affected family members of two of the patients were found to have the same mutations as the patients. Selected candidate genes were analyzed in patients with stress fractures to estimate the significance of these genes in the pathogenesis of stress factors. No disease-causing, or predisposing variations were found in the genes. However, an association between a certain *VDR* haplotype and femoral neck stress fractures was found. It accounted for a five-fold increase in the risk of stress fractures, with an associated attributable risk of 12%.

The results showed that genetic factors play a role in different bone phenotypes. The finding that patients with OI, a monogenic disorder, have mutations in the collagen I genes was not novel. However, the lack of correlation between a genotype and the clinical phenotype in OI suggests that, even in a monogenic disorder, additional genetic factors have an influence on the phenotypic outcome. This is also supported by the finding that an identical mutation can result in different phenotypes, even in members of the same family. OI overlaps clinically with juvenile osteoporosis. Despite this, no mutations were found in the collagen I genes in 20 patients with JIO. Instead, the analysis of *LRP5* revealed two missense mutations and one frameshift mutation in three of the patients. Two of the heterozygous mutations were also observed in the affected family members, who all had osteoporosis. These results suggest that heterozygous *LRP5* mutations may also play a role in the general adult population with osteoporosis. It was

also shown that genetic variations play a role in developing stress fractures. Candidate gene analyses of five different genes, and association analyses of 11 polymorphisms, revealed no mutations, or predisposing variations. Instead, a highly significant association was found between the phenotype and a VDR haplotype that accounts for a five-fold increase in the risk of stress fractures.

This study of genetic factors in OI, JIO and stress fractures provided new information concerning genotype-phenotype correlations, identified disease-causing and predisposing genetic variations in these disorders. These findings provide new information on the pathogenesis of the disorders, which will be useful for the development of genetic testing and targeted treatment for the disorders.

## References

- Anderson MW & Greenspan A (1996) Stress fractures. *Radiology* 199: 1-12.
- Arai H, Miyamoto K, Taketani Y, Yamamoto H, Iemori Y, Morita K, Tonai T, Nishisho T, Mori S & Takeda E (1997) A vitamin D receptor gene polymorphism in the translation initiation codon: effect on protein activity and relation to bone mineral density in Japanese women. *J Bone Miner Res* 12: 915-921.
- Babij P, Zhao W, Small C, Kharode Y, Yaworsky PJ, Boussein ML, Reddy PS, Bodine PV, Robinson JA, Bhat B, Marzolf J, Moran RA & Bex F (2003) High bone mass in mice expressing a mutant LRP5 gene. *J Bone Miner Res* 18: 960-974.
- Barr FG, Galili N, Holick J, Biegel JA, Rovera G & Emanuel BS (1993) Rearrangement of the PAX3 paired box gene in the paediatric solid tumour alveolar rhabdomyosarcoma. *Nat Genet* 3: 113-117.
- Barsh GS, Roush CL, Bonadio J, Byers PH & Gelinas RE (1985) Intron-mediated recombination may cause a deletion in an  $\alpha 1$  type I collagen chain in a lethal form of osteogenesis imperfecta. *Proc Natl Acad Sci USA* 82: 2870-2874.
- Beighton P, Winship I & Behari D (1985) The ocular form of osteogenesis imperfecta: a new autosomal recessive syndrome. *Clin Genet* 28: 69-75.
- Berger G, Hawke M, Johnson A & Proops D (1985) Histopathology of the temporal bone in osteogenesis imperfecta congenita: a report of 5 cases. *Laryngoscope* 95: 193-199.
- Bergstrom L (1981) Fragile bones and fragile ears. *Clin Orthop Relat Res* 159: 58-63.
- Birchall JP, Pearman K & Dawes JD (1982) The collagens of middle-ear structures and tympanosclerotic plaques. *J Laryngol Otol* 96: 797-800.
- Blair HC, Kahn AJ, Crouch EC, Jeffrey JJ & Teitelbaum SL (1986) Isolated osteoclasts resorb the organic and inorganic components of bone. *J Cell Biol* 102: 1164- 1172.
- Bollerslev J & Andersen PE Jr (1988) Radiological, biochemical and hereditary evidence of two types of autosomal dominant osteopetrosis. *Bone* 9: 7-13.
- Bono CM & Einhorn TA (2003) Overview of osteoporosis: pathophysiology and determinants of bone strength. *Eur Spine J* 12 (Suppl. 2): 90-96.
- Boyden LM, Mao J, Belsky J, Mitzner L, Farhi A, Mitnick MA, Wu D, Insogna K & Lifton RP (2002) High bone density due to a mutation in LDL-receptor-related protein 5. *N Engl J Med* 346: 1513-1521.
- Braga V, Sangalli A, Malerba G, Mottes M, Mirandola S, Gatti D, Rossini M, Zamboni M & Adami S (2002) Relationship among VDR (BsmI and FokI), COL1A1, and CTR polymorphisms with bone mass, bone turnover markers, and sex hormones in men. *Calcif Tissue Int* 70: 457-462.
- Breithaupt (1855) Zur Pathologie menschlichen Fusses. *Med Zeitung* 24: 169-175.

- Brown SD, Twells RC, Hey PJ, Cox RD, Levy ER, Soderman AR, Metzker ML, Caskey CT, Todd JA & Hess JF (1998) Isolation and characterization of LRP6, a novel member of the low density lipoprotein receptor gene family. *Biochem Biophys Res Commun* 248: 879-888.
- Brude E (1986) Ocular osteogenesis imperfecta. *Clin. Genet* 29: 187.
- Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL, Boyle WJ & Simonet WS (1998) Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 12: 1260-1268.
- Burger EH & Klein-Nulend J (1999) Mechanotransduction in bone--role of the lacuno-canalicular network. *FASEB J* 13 (Suppl. 1) S101-S112.
- Burr DB, Milgrom C, Boyd RD, Higgins WL, Robin G & Radin EL (1990) Experimental stress fractures of the tibia. Biological and mechanical aetiology in rabbits. *J Bone Joint Surg Br* 72: 370-375.
- Burr DB (2001) Rabbits as an animal model for stress fractures. In: Burr DB & Milgrom C (eds) *Musculoskeletal fatigue and stress fractures*. CRC Press LLC, Florida p. 221-232.
- Byers PH, Tsipouras P, Bonadio JF, Starman BJ & Schwartz RC (1988) Perinatal lethal osteogenesis imperfecta (OI type II): a biochemically heterogeneous disorder usually due to new mutations in the gene for type I collagen. *Am J Hum Genet* 42: 237-248.
- Byers PH (1990) Brittle bones--fragile molecules: disorders of collagen gene structure and expression. *Trends Genet* 6: 293-300.
- Byers PH, Wallis GA & Willing MC (1991) Osteogenesis imperfecta: translation of mutation to phenotype. *J Med Genet*. 28: 433-442.
- Byers PH (2000) Osteogenesis imperfecta: perspectives and opportunities. *Curr Opin Pediatr* 12: 603-609.
- Byers PH & Cole WG (2002) Osteogenesis imperfecta. In: Royce PM & Steinmann B (eds). *Connective Tissue and Its Heritable Disorders: Molecular, Genetic, and Medical Aspects*. Wiley-Liss, New York p. 385-430.
- Cartegni L, Chew SL & Krainer AR (2002) Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat Rev Genet* 3: 285-298.
- Chen WJ, Goldstein JL & Brown MS (1990) NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J Biol Chem* 265: 3116-3123.
- Chiano MN & Clayton DG (1998) Fine genetic mapping using haplotype analysis and the missing data problem. *Ann Hum Genet* 62: 55-60.
- Chipman SD, Sweet HO, McBride DJ Jr, Davison MT, Marks SC Jr, Shuldiner AR, Wenstrup RJ, Rowe DW & Shapiro JR (1993) Defective pro alpha 2(I) collagen synthesis in a recessive mutation in mice: a model of human osteogenesis imperfecta. *Proc Natl Acad Sci USA* 90: 1701-1705.
- Chu ML, Williams CJ, Pepe G, Hirsch JL, Prockop DJ & Ramirez F (1983) Internal deletion in a collagen gene in a perinatal lethal form of osteogenesis imperfecta. *Nature* 304: 78-80.
- Chu ML, de Wet W, Bernard M, Ding JF, Morabito M, Myers J, Williams C & Ramirez F (1984) Human pro alpha 1(I) collagen gene structure reveals evolutionary conservation of a pattern of introns and exons. *Nature* 310: 337-340.
- Cleiren E, Benichou O, Van Hul E, Gram J, Bollerslev J, Singer FR, Beaverson K, Aledo A, Whyte M P, Yoneyama T, deVernejoul MC & Van Hul W (2001) Albers-Schonberg disease (autosomal dominant osteopetrosis, type II) results from mutations in the CICN7 chloride channel gene. *Hum Molec Genet* 10: 2861-2867.
- Consensus Development Conference (1993) Diagnosis, prophylaxis, and treatment of osteoporosis *Am J Med* 94: 646-50.
- Dawson PA, Kelly TE & Marini JC (1999) Extension of phenotype associated with structural mutations in type I collagen: siblings with juvenile osteoporosis have an alpha2(I)Gly436 --> Arg substitution. *J Bone Miner Res* 14: 449-455.
- Dent CE & Friedman M (1965) Idiopathic juvenile osteoporosis *Q J Med* 34: 177-210.
- Dickson IR, Gwilliam R, Arora M, Murphy S, Khaw KT, Phillips C & Lincoln P (1994) Lumbar vertebral and femoral neck bone mineral density are higher in postmenopausal women with the alpha 2HS-glycoprotein 2 phenotype. *Bone Miner* 24: 181-188.

- Dieler R, Muller J & Helms J (1997) Stapes surgery in osteogenesis imperfecta patients. *Eur Arch Otorhinolaryngol* 254: 120-127.
- Ducy P & Karsenty G (1996) Skeletal gla proteins: gene structure, regulation of expression, and function. In: Bilezikian JP, Raisz LG & Rodan GA (eds) *Principles of bone biology*. Academic Press, Inc, San Diego, California, p. 183-196.
- Ducy P, Schinke T & Karsenty G (2000) The osteoblast: a sophisticated fibroblast under central surveillance. *Science* 289: 1501-1504.
- Eccleshall TR, Garnero P, Gross C, Delmas PD & Feldman D (1998) Lack of correlation between start codon polymorphism of the vitamin D receptor gene and bone mineral density in premenopausal French women: the OFELY study. *J Bone Miner Res* 13: 31-35.
- Engel J & Prockop DJ (1991) The zipper-like folding of collagen triple helices and the effects of mutations that disrupt the zipper. *Annu Rev Biophys Biophys Chem* 20: 137-152.
- Eriksen EF (1986) Normal and pathological remodelling of human trabecular bone: three dimensional reconstruction of the remodelling sequence in normals and in metabolic bone disease. *Endocr Rev* 7: 379-408.
- Faraco JH, Morrison NA, Baker A, Shine J & Frossard PM (1989) Apal dimorphism at the human vitamin D receptor gene locus. *Nucleic Acids Res* 17: 2150.
- Faustino NA & Cooper TA (2003) Pre-mRNA splicing and human disease. *Genes Dev* 17: 419-437.
- Ferrari S, Rizzoli R, Manen D, Slosman D & Bonjour JP (1998) Vitamin D receptor gene start codon polymorphisms (FokI) and bone mineral density: interaction with age, dietary calcium, and 3'-end region polymorphisms. *J Bone Miner Res* 13: 925-930.
- Ferrari SL, Deutsch S, Choudhury U, Chevalley T, Bonjour J-P, Dermitzakis ET, Rizzoli R & Antonarakis SE (2004) Polymorphisms in the low-density lipoprotein receptor-related protein 5 (LRP5) gene are associated with variation in vertebral bone mass, vertebral bone size, and stature in whites. *Am J Hum Genet.* 74: 866-875.
- Fisher LW, Termine JD & Young MF (1989) Deduced protein sequence of bone small proteoglycan I (biglycan) shows homology with proteoglycan II (decorin) and several nonconnective tissue proteins in a variety of species. *J Biol Chem.* 264: 4571-4576.
- Forlino A, Porter FD, Lee EJ, Westphal H & Marini JC (1999) Use of the Cre/lox recombination system to develop a non-lethal knock-in murine model for osteogenesis imperfecta with an alpha1(I) G349C substitution. Variability in phenotype in BrlIV mice. *J Biol Chem* 274: 37923-37931.
- Friedman E, Vered I & Shemer J (2001) The genetic basis for stress fractures. In: Burr DB & Milgrom C (eds) *Musculoskeletal fatigue and stress fractures*. CRC Press LLC, Florida p. 105-118.
- Furie B & Furie BC (1988) The molecular basis of blood coagulation. *Cell* 53: 505-518.
- Ganguly A, Rock MJ & Prockop DJ (1993) Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. *Proc Natl Acad Sci USA* 90: 10325-10329.
- Ganguly A (2002) An update on conformation sensitive gel electrophoresis. *Hum Mutat* 19: 334-342.
- Garretsen AJ, Cremers CW & Huygen PL (1997) Hearing loss (in nonoperated ears) in relation to age in osteogenesis imperfecta type I. *Ann Otol Rhinol Laryngol* 106: 575-582.
- Glorieux FH, Rauch F, Plotkin H, Ward L, Travers R, Roughley P, Lalic L, Glorieux DF, Fassier F & Bishop NJ (2000) Type V osteogenesis imperfecta: a new form of brittle bone disease. *J Bone Miner Res* 15: 1650-1658.
- Glorieux FH, Ward LM, Rauch F, Lalic L, Roughley PJ & Travers R (2002) Osteogenesis imperfecta type VI: a form of brittle bone disease with a mineralization defect. *J Bone Miner Res* 17: 30-38.

- Gong Y, Vikkula M, Boon L, Liu J, Beighton P, Ramesar R, Peltonen L, Somer H, Hirose T, Dallapiccola B, De Paepe A, Swoboda W, Zabel B, Superti-Furga A, Steinmann B, Brunner HG, Jans A, Boles RG, Adkins W, van den Boogaard MJ, Olsen BR & Warman ML (1996) Osteoporosis-pseudoglioma syndrome, a disorder affecting skeletal strength and vision, is assigned to chromosome region 11q12-13. *Am J Hum Genet* 59: 146-151.
- Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D, Zacharin M, Oexle K, Marcelino J, Suwairi W, Heeger S, Sabatakos G, Apte S, Adkins WN, Allgrove J, Arslan-Kirchner M, Batch JA, Beighton P, Black GC, Boles RG, Boon LM, Borrone C, Brunner HG, Carle GF, Dallapiccola B, De Paepe A, Floege B, Halfhide ML, Hall B, Hennekam RC, Hirose T, Jans A, Juppner H, Kim CA, Keppler-Noreuil K, Kohlschuetter A, LaCombe D, Lambert M, Lemyre E, Letteboer T, Peltonen L, Ramesar RS, Romanengo M, Somer H, Steichen-Gersdorf E, Steinmann B, Sullivan B, Superti-Furga A, Swoboda W, van den Boogaard MJ, Van Hul W, Vikkula M, Votruba M, Zabel B, Garcia T, Baron R, Olsen BR & Warman ML (2001) LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell* 107: 513-523.
- Grant SF, Reid DM, Blake G, Herd R, Fogelman I & Ralston SH (1996) Reduced bone density and osteoporosis associated with a polymorphic Sp1 binding site in the collagen type I alpha 1 gene. *Nat Genet* 14: 203-205.
- Gross C, Eccleshall TR, Malloy PJ, Villa ML, Marcus R & Feldman D (1996) The presence of a polymorphism at the translation initiation site of the vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican-American women. *J Bone Miner Res* 11: 1850-1855.
- Grzesik WJ & Robey PG (1994) Bone matrix RGD glycoproteins: immunolocalization and interaction with human primary osteoblastic bone cells in vitro. *J Bone Miner Res* 9: 487-496.
- Hale JE, Fraser JD & Price PA (1988) The identification of matrix Gla protein in cartilage. *J Biol Chem* 263: 5820-5824.
- Hall JG (1993) The clinical behavior of hereditary syndromes, with a precis of medical genetics. In: Beighton P (ed) *McKusick's Heritable disorders of connective tissue*. Mosby-Year Book, Saint Louis p. 1-32.
- Hanke M & Wink M (1994) Direct DNA sequencing of PCR-amplified vector inserts following enzymatic degradation of primer and dNTPs. *Biotechniques* 17: 858-860.
- Hanscom DA & Bloom BA (1988) The spine in osteogenesis imperfecta. *Orthop Clin North Am* 19: 449-458.
- Hauschka PV, Lian JB, Cole DE & Gundberg CM (1989) Osteocalcin and matrix Gla protein: vitamin K-dependent proteins in bone. *Physiol Rev* 69: 990-1047.
- Haussler MR, Whitfield GK, Haussler CA, Hsieh JC, Thompson PD, Selznick SH, Dominguez CE & Jurutka PW (1998) The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J Bone Miner Res* 13: 325-349.
- He X, Semenov M, Tamai K & Zeng X (2004) LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signalling: arrows point the way. *Development* 131: 1663-1677.
- Heinegård D, Aspberg A, Franzén A & Lorenzo P (2002) Glycosylated matrix proteins. In: Royce PM & Steinmann B (eds). *Connective Tissue and Its Heritable Disorders: Molecular, Genetic, and Medical Aspects*. Wiley-Liss, Inc, New York p. 271-291.
- Heller S, Sheane CA, Javed Z & Hudspeth AJ (1998) Molecular markers for cell types of the inner ear and candidate genes for hearing disorders. *Proc Natl Acad Sci U S A* 95: 11400-11405.
- Hey PJ, Twells RC, Phillips MS, Yusuke Nakagawa, Brown SD, Kawaguchi Y, Cox R, Guochun Xie, Dugan V, Hammond H, Metzker ML, Todd JA & Hess JF (1998) Cloning of a novel member of the low-density lipoprotein receptor family. *Gene* 216: 103-111.
- Houston LA, Grant SFA, Reid DM & Ralston SH (1996) Vitamin D receptor polymorphism, bone mineral density, and osteoporotic vertebral fracture: Studies in a UK population. *Bone* 18: 249-252.
- Huerre C, Junien C, Weil D, Chu ML, Morabito M, Van Cong N, Myers JC, Foubert C, Gross MS, Prockop DJ, Boue A, Kaplan JC, de la Chapelle A & Ramirez F (1982) Human type I procollagen genes are located on different chromosomes. *Proc Natl Acad Sci USA* 79: 6627-6630.

- Hughes AE, Ralston SH, Marken J, Bell C, MacPherson H, Wallace RGH, van Hul W, Whyte MP, Nakatsuka K, Hovy L & Anderson DM (2000) Mutations in TNFRSF11A, affecting the signal peptide of RANK, cause familial expansile osteolysis. *Nature Genet* 24: 45-48.
- Hughes DE (2000) Osteoarticular and connective tissues. In: Underwood JCE (ed) *General and systematic pathology*. Harcourt Publishers limited, London p. 699-736.
- Hughes MR, Malloy PJ, Kieback DG, Kesterson RA, Pike JW, Feldman D & O'Malley BW (1988) Point mutations in the human vitamin D receptor gene associated with hypocalcemic rickets. *Science* 242: 1702-1705.
- Hustmyer FG, Peacock M, Hui S, Johnston CC & Christian J (1994) Bone mineral density in relation to polymorphism at the vitamin D receptor gene locus. *J Clin Invest* 94: 2130-2134.
- Janssens K & Van Hul W (2002) Molecular genetics of too much bone *Hum Mol Genet* 11: 2385-2393.
- Johnell O, Scheele WH, Lu Y, Reginster JY, Need AG & Seeman E (2002) Additive effects of raloxifene and alendronate on bone density and biochemical markers of bone remodelling in postmenopausal women with osteoporosis. *J Clin Endocrinol Metab* 87: 985-992.
- Johnson ML, Gong G, Kimberling W, Recker SM, Kimmel DB & Recker RB (1997) Linkage of a gene causing high bone mass to human chromosome 11 (11q12-13) *Am J Hum Genet* 60: 1326-1332.
- Jones BH, Thacker SB, Gilchrist J, Kimsey CD & Sosin DM (2002) Prevention of lower extremity stress fractures in athletes and soldiers: a systematic review. *Epidemiol Rev* 24: 228-247.
- Kahraman H, Duman BS, Alagol F, Tanakol R & Yilmazer S (2004) Lack of association between vitamin D receptor gene polymorphism (BsmI) and osteomalacia. *J Bone Miner Metab* 22: 39-43.
- Kanis JA (2002) Diagnosis of osteoporosis and assessment of fracture risk. *Lancet* 359: 1929-1936.
- Kato M, Patel MS, Lévassieur R, Lobov I, Chang BH, Glass DA 2nd, Hartmann C, Li L, Hwang TH, Brayton CF, Lang RA, Karsenty G & Chan L (2002) Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. *J Cell Biol* 157: 303-314.
- Khillan JS, Olsen AS, Kontusaari S, Sokolov B & Prockop DJ (1991) Transgenic mice that express a mini-gene version of the human gene for type I procollagen (COL1A1) develop a phenotype resembling a lethal form of osteogenesis imperfecta. *J Biol Chem* 266: 23373-23379.
- Kiuru MJ, Pihlajamäki HK & Ahovuo JA (2004) Bone stress injuries. *Acta Radiol* 45: 317-326.
- Kivirikko KI (1993) Collagens and their abnormalities in a wide spectrum of diseases. *Ann Med* 25: 113-126.
- Klein, RM & Norman A (1995) Diagnostic procedures for Paget's disease: radiologic, pathologic, and laboratory testing. *Endocr Metab Clin North Am* 24: 437-450.
- Kornak U, Schulz A, Friedrich W, Uhlhaas S, Kremens B, Voit T, Hasan C, Bode U, Jentsch TJ & Kubisch C (2000) Mutations in the  $\alpha 3$  subunit of the vacuolar H(+)-ATPase cause infantile malignant osteopetrosis. *Hum Mol Genet* 9: 2059-2063.
- Krieger M & Herz J (1994) Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu Rev Biochem* 63: 601-637.
- Krust A, Green S, Argos P, Kumar V, Walter P, Bornert JM & Chambon P (1986) The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocorticoid receptors. *EMBO J* 5: 891-897.
- Kuivaniemi H, Tromp G & Prockop DJ (1991) Mutations in collagen genes: causes of rare and some common diseases in humans. *FASEB J* 5: 2052-2060.
- Kuivaniemi H, Tromp G & Prockop DJ (1997) Mutations in fibrillar collagens (types I, II, III and XI), fibril-associated collagen (type IX), and network-forming collagen (type X) cause a spectrum of diseases of bone, cartilage and blood vessels. *Hum Mutat* 9: 300-315.
- Kuurila K, Kaitila I, Johansson R & Grenman R (2002) Hearing loss in Finnish adults with osteogenesis imperfecta: a nationwide survey. *Ann Otol Rhinol Laryngol* 111: 939-946.
- Kuznetsova NV, Forlino A, Cabral WA, Marini JC & Leikin S (2004) Structure, stability and interactions of type I collagen with GLY349-CYS substitution in alpha 1(I) chain in a murine Osteogenesis Imperfecta model. *Matrix Biol* 23: 101-112.

- Körkkö J, Ala-Kokko L, De Paepe A, Nuytincq L, Earley J & Prockop DJ (1998a) Analysis of the COL1A1 and COL1A2 genes by PCR amplification and scanning by conformation-sensitive gel electrophoresis identifies only COL1A1 mutations in 15 patients with osteogenesis imperfecta type I: identification of common sequences of null-allele mutations. *Am J Hum Genet* 62: 98-110.
- Körkkö J, Annunen S, Pihlajamaa T, Prockop DJ & Ala-Kokko L (1998b) Conformation sensitive gel electrophoresis for simple and accurate detection of mutations: comparison with denaturing gradient gel electrophoresis and nucleotide sequencing. *Proc Natl Acad Sci USA* 95: 1681-1685.
- Körkkö J, Kaitila I, Lonnqvist L, Peltonen L & Ala-Kokko L (2002) Sensitivity of conformation sensitive gel electrophoresis in detecting mutations in Marfan syndrome and related conditions. *J Med Genet* 39: 34-41.
- Laaksonen M, Karkkainen M, Outila T, Vanninen T, Ray C & Lamberg-Allardt C (2002) Vitamin D receptor gene BsmI-polymorphism in Finnish premenopausal and postmenopausal women: its association with bone mineral density, markers of bone turnover, and intestinal calcium absorption, with adjustment for lifestyle factors. *J Bone Miner Metab* 20: 383-390.
- Lalwani AK, Mhatre AN, San Agustin TB & Wilcox ER (1996) Genotype-phenotype correlations in type I Waardenburg syndrome. *Laryngoscope* 106: 895-902.
- Lambros G & Alder D (1997) Multiple stress fractures of the tibia in a healthy adult. *Am J Orthop* 26: 687-688.
- Langdahl BL, Gravholt CH, Brixen K & Eriksen EF (2000) Polymorphisms in the vitamin D receptor gene and bone mass, bone turnover and osteoporotic fractures. *Eur J Clin Invest* 30: 608-617.
- Laurin N, Brown JP, Morissette J & Raymond V (2002) Recurrent mutation of the gene encoding sequestosome 1 (SQSTM1/p62) in Paget disease of bone. *Am J Hum Genet* 70: 1582-1588.
- Lei SF, Deng FY, Liu XH, Huang QR, Qin Y, Zhou Q, Jiang DK, Li YM, Mo XY, Liu MY, Chen XD, Wu XS, Shen H, Dvornyk V, Zhao L, Recker RR & Deng HW (2003) Polymorphisms of four bone mineral density candidate genes in Chinese populations and comparison with other populations of different ethnicity. *J Bone Miner Metab* 21: 34-42.
- Levin LS, Salinas CF & Jorgenson RJ (1978) Classification of osteogenesis imperfecta by dental characteristics. *Lancet* 1: 322.
- Li G, Zhang S, Chen G, Chen H & Wang AM (1985) Radiographic and histologic analysis of stress fracture in a rabbit tibias. *Am J Sports Med* 13: 285-294.
- Liberman UA (1996) Hereditary deficiencies in vitamin D action. In: Bilezikian JP, Raisz LG & Rodan GA (eds) *Principles of bone biology*. Academic Press, Inc, San Diego, California p. 903-916.
- Lim SK, Park YS, Park JM, Song YD, Lee EJ, Kim KR, Lee HC & Huh KB (1995) Lack of association between vitamin D receptor genotypes and osteoporosis in Koreans. *J Clin Endocrinol Metab* 80: 3677-3681.
- Lindsay R (1998) The role of estrogen in the prevention of osteoporosis. *Endocrinol Metab Clin North Am* Jun 27: 399-409.
- Little RD, Carulli JP, Del Mastro RG, Dupuis J, Osborne M, Folz C, Manning SP, Swain PM, Zhao SC, Eustace B, Lappe MM, Spitzer L, Zweier S, Braunschweiger K, Benchekroun Y, Hu X, Adair R, Chee L, FitzGerald MG, Tulig C, Caruso A, Tzellas N, Bawa A, Franklin B, McGuire S, Nogues X, Gong G, Allen KM, Anisowicz A, Morales AJ, Lomedico PT, Recker SM, Van Eerdewegh P, Recker RR & Johnson ML (2002) A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. *Am J Hum Genet* 70: 11-19.
- Lorenc RS (2002) Idiopathic juvenile osteoporosis. *Calcif Tissue Int* 70: 395-397.
- Loria-Cortés R, Quesada-Calvo E & Cordero-Chaverri C (1977) Osteopetrosis in children: a report of 26 cases. *J Pediatr* 91: 43-47.
- MacDonald PN, Dowd DR, Nakajima S, Galligan MA, Reeder MC, Haussler CA, Ozato K & Haussler MR (1993) Retinoid X receptors stimulate and 9-*cis* retinoic acid inhibits, 1,25-dihydroxyvitamin D<sub>3</sub>-activated expression of the rat osteocalcin gene. *Mol Cell Biol* 13: 5907-5917.

- Mackie EJ (2003) Osteoblasts: novel roles in orchestration of skeletal architecture. *Int J Biochem Cell Biol* 35: 1301-1305.
- Malloy P, Eccleshall T, Gross C, Van Maldergem L, Bouillon R & Feldman D (1997) Hereditary vitamin D resistant rickets caused by a novel mutation in the vitamin D receptor that results in decreased affinity for hormone and cellular hyporesponsiveness. *J Clin Invest* 99: 297-304.
- Mann V, Hobson EE, Li B, Stewart TL, Grant SF, Robins SP, Aspden RM & Ralston SH (2001) A COL1A1 Sp1 binding site polymorphism predisposes to osteoporotic fracture by affecting bone density and quality. *J Clin Invest* 107: 899-907.
- Mann V & Ralston SH (2003) Meta-analysis of COL1A1 Sp1 polymorphism in relation to bone mineral density and osteoporotic fracture. *Bone* 32: 711-717.
- Mao B, Wu W, Li Y, Hoppe D, Stanek P, Glinka A & Niehrs C (2001a) LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature* 411: 321-325.
- Mao J, Wang J, Liu B, Pan W, Farr GH 3rd, Flynn C, Yuan H, Takada S, Kimelman D, Li L & Wu D (2001b) Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. *Mol Cell* 7: 801-809.
- Marlowe A, Pepin MG & Byers PH (2002) Testing for osteogenesis imperfecta in cases of suspected non-accidental injury. *J Med Genet* 39: 382-386.
- Marks SC & Hermey DC (1996) The structure and development of bone. In: Bilezikian JP, Raisz LG & Rodan GA (eds) *Principles of bone biology*. Academic Press, Inc, San Diego, California p. 155-166.
- McGuigan FE, Murray L, Gallagher A, Davey-Smith G, Neville CE, Van't Hof R, Boreham C & Ralston SH (2002) Genetic and environmental determinants of peak bone mass in young men and women. *J Bone Miner Res* 17: 1273-1279.
- Mellstrom DD, Sorensen OH, Goemaere S, Roux C, Johnson TD, & Chines AA (2004) Seven Years of Treatment with Risedronate in Women with Postmenopausal Osteoporosis. *Calcif Tissue Int Oct* 7.
- Melton LJ III (1995) How many women have osteoporosis now? *J Bone Miner Res* 10: 298-305.
- Milgrom C, Chisin R, Giladi M, Stein M, Kashtan H, Margulies J & Atlan H (1985) Multiple stress fractures. A longitudinal study of a soldier with 13 lesions. *Clin Orthop* 192: 174-179.
- Mills BG & Singer FR (1976) Nuclear inclusions in Paget's disease of bone. *Science* 194: 201-202.
- Miyamoto K, Kesterson RA, Yamamoto H, Taketani Y, Nishiwaki E, Tatsumi S, Inoue Y, Morita K, Takeda E & Pike JW (1997) Structural organization of the human vitamin D receptor chromosomal gene and its promoter. *Mol Endocrinol* 11: 1165-1179.
- Mizuguchi T, Furuta I, Watanabe Y, Tsukamoto K, Tomita H, Tsujihata M, Ohta T, Kishino T, Matsumoto N, Minakami H, Niikawa N & Yoshiura K (2004) LRP5, low-density-lipoprotein-receptor-related protein 5, is a determinant for bone mineral density. *J Hum Genet* 49: 80-86.
- Mori S & Burr DB (1993) Increased intracortical remodelling following fatigue damage. *Bone* 14: 103-109.
- Mori S, Li J & Kawaguchi Y (2001) The histological appearance of stress fractures. In: Burr DB & Milgrom C (eds) *Musculoskeletal fatigue and stress fractures*. CRC Press LLC, Florida p. 151-160.
- Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN & Eisman JA (1994) Prediction of bone density from vitamin D receptor alleles. *Nature* 367: 284-287.
- Mylyharju J & Kivirikko KI (2004) Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet* 20: 33-43.
- Nakamura M, Zhang ZQ, Shan L, Hisa T, Sasaki M, Tsukino R, Yokoi T, Kaname A & Kakudo K (1997) Allelic variants of human calcitonin receptor in the Japanese population. *Hum Genet* 99: 38-41.
- Nance WE (2003) The genetics of deafness. *Ment Retard Dev Disabil Res Rev* 9: 109-119.
- Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster JY, Hodsmann AB, Eriksen EF, Ish-Shalom S, Genant HK, Wang O & Mitlak BH (2001) Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N Engl J Med* 344: 1434-1441.

- Nicholls AC, Osse G, Schloos HG, Lenard HG, Deak S, Myers JC, Prockop DJ, Weigel WR, Fryer P & Pope FM (1984) The clinical features of homozygous alpha 2(I) collagen deficient osteogenesis imperfecta. *J Med Genet* 21: 257-262.
- Nielens H, Devogelaer JP & Malghem J (1994) Occurrence of a painful stress fracture of the femoral neck simultaneously with six other asymptomatic localizations in a runner. *J Sports Med Phys Fitness* 34: 79-82.
- Norman AW & Collins ED (1996) Vitamin D receptor structure, expression, and nongenomic effects. In: Bilezikian JP, Raisz LG & Rodan GA (eds) *Principles of bone biology*. Academic Press, Inc, San Diego, California p. 419-434.
- Oldberg A, Antonsson P, Lindblom K & Heinegard D (1989) A collagen-binding 59-kd protein (fibromodulin) is structurally related to the small interstitial proteoglycans PG-S1 and PG-S2 (decorin). *EMBO J* 8: 2601-2604.
- Pace JM, Atkinson M, Willing MC, Wallis G & Byers PH (2001) Deletions and duplications of Gly-Xaa-Yaa triplet repeats in the triple helical domains of type I collagen chains disrupt helix formation and result in several types of osteogenesis imperfecta. *Hum Mutat* 18: 319-326.
- Patel MS & Karsenty G (2002) Regulation of bone formation and vision by LRP5. *N Engl J Med* 346: 1572-1574.
- Paterson CR, Monk EA, & McAllion SJ (2001) How common is hearing impairment in osteogenesis imperfecta? *J Laryngol Otol* 115: 280-282.
- Peacock M, Turner CH, Econs MJ & Foroud T (2002) Genetics of osteoporosis. *Endocr Rev* 23: 303-326.
- Pedersen U (1984) Hearing loss in patients with osteogenesis imperfecta. A clinical and audiological study of 201 patients. *Scand Audiol* 13: 67-74.
- Pepin M, Atkinson M, Starman BJ & Byers PH (1997) Strategies and outcomes of prenatal diagnosis for osteogenesis imperfecta: a review of biochemical and molecular studies completed in 129 pregnancies. *Prenat Diagn* 17: 559-570.
- Pihlajaniemi T, Dickson LA, Pope FM, Korhonen VR, Nicholls A, Prockop DJ & Myers JC (1984) Osteogenesis imperfecta: cloning of a pro-alpha 2(I) collagen gene with a frameshift mutation. *J Biol Chem* 259: 12941-12944.
- Pinson KI, Brennan J, Monkley S, Avery BJ & Skarnes WC (2000) An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* 407: 535-538.
- Pocock NA, Eisman JA, Hopper JL, Yeates MG, Sambrook PN & Eberl S (1987) Genetic determinants of bone mass in adults: a twin study. *J Clin Invest* 80: 706-710.
- Price PA, Fraser JD & Metz-Virca G. (1987) Molecular cloning of matrix Gla protein: implications for substrate recognition by the vitamin K-dependent gamma-carboxylase. *Proc Natl Acad Sci USA* 84: 8335-8339.
- Prockop DJ & Kivirikko KI (1984) Heritable diseases of collagen. *N Engl J Med* 31: 376-386.
- Prockop DJ, Kuivaniemi H & Tromp G (1994) Molecular basis of osteogenesis imperfecta and related disorders of bone. *Clin Plast Surg*. 21: 407-413.
- Prockop DJ & Kivirikko KI (1995) Collagens: Molecular biology, diseases and potentials for therapy. *Annu Rev Biochem* 64: 403-443.
- Radmer RJ & Klein TE (2004) Severity of osteogenesis imperfecta and structure of a collagen-like peptide modeling a lethal mutation site. *Biochemistry* 43: 5314-5323.
- Ralston SH (2002) Genetic control of susceptibility to osteoporosis. *J Clin Endocrinol Metab* 87: 2460-2466.
- Rauch F, Travers R, Norman ME, Taylor A, Parfitt AM & Glorieux FH (2000) Deficient bone formation in idiopathic juvenile osteoporosis: a histomorphometric study of cancellous iliac bone. *J Bone Miner Res* 15: 957-963.
- Rauch F, Travers R, Norman ME, Taylor A, Parfitt AM & Glorieux FH (2002) The bone formation defect in idiopathic juvenile osteoporosis is surface-specific. *Bone* 31: 85-89.
- Rauch F & Glorieux FH (2004) Osteogenesis imperfecta. *Lancet* 363: 1377-1385.
- Ravine D & Cooper DN (1997) Adult-onset genetic disease: mechanisms, analysis and prediction. *QJM* 90: 83-103.

- Rawadi G, Vayssiere B, Dunn F, Baron R & Roman-Roman S (2003) BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. *J Bone Miner Res* 18: 1842-1853.
- Riggs BL & Melton LJ (1983) Evidence for two distinct syndrome of involuntional osteoporosis. *Am J Med* 309: 899-901.
- Riggs BL, Nguyen TV, Melton LJ, Morrison NA, O'Fallon WM, Kelly PJ, Egan KS, Sambrook PN, Muhs JM & Eisman JA (1995) The contribution of vitamin D receptor gene alleles to the determination of bone mineral density in normal and osteoporotic women. *J Bone Miner Res* 10: 991-996.
- Robertson NG, Khetarpal U, Gutierrez-Espeleta GA, Bieber FR & Morton CC (1982) Isolation of novel and known genes from a human fetal cochlear cDNA library using subtractive hybridization and differential screening. *Genomics* 23: 42-50.
- Robey PG (1996) Bone matrix proteoglycans and glycoproteins. In: Bilezikian JP, Raisz LG & Rodan GA (eds) *Principles of bone biology*. Academic Press, Inc, San Diego, California p. 155-166.
- Robinow M (1985) Osteoporosis-pseudoglioma syndrome? *Clin. Genet* 28: 359.
- Rossert J & de Crombrugge B (1996) Type I collagen: structure, synthesis, and regulation. In: Bilezikian JP, Raisz LG & Rodan GA (eds) *Principles of bone biology*. Academic Press, Inc, San Diego, California p. 127-142.
- Schaffler MB (2001) Bone fatigue and remodelling in the development of stress fractures. In: Burr DB & Milgrom C (eds) *Musculoskeletal fatigue and stress fractures*. 1<sup>st</sup> ed. CRC Press LLC, Florida p. 161-182.
- Schenk RK, Hofstetter W & Felix R (2002) Bone. In: Royce PM & Steinmann B (eds) *Connective Tissue and Its Heritable Disorders: Molecular, Genetic, and Medical Aspects*. Wiley-Liss, New York p. 67-120.
- Schnieke A, Harbers K & Jaenisch R (1983) Embryonic lethal mutation in mice induced by retrovirus insertion into the alpha 1(I) collagen gene. *Nature* 304: 315-320.
- Schulte (1897) Die sogenannte Fussgeschwulst. *Arch Klin Chir* 55: 872.
- Schwarze U, Hata R, McKusick VA, Shinkai H, Hoyme HE, Pyeritz RE & Byers PH (2004) Rare autosomal recessive cardiac valvular form of Ehlers-Danlos syndrome results from mutations in the COL1A2 gene that activate the nonsense-mediated RNA decay pathway. *Am J Hum Genet* 74: 917-930.
- Seeman E (2002) Pathogenesis of bone fragility in women and men. *Lancet* 359: 1841-1850.
- Seeman E (2003) Reduced bone formation and increased bone resorption: rational targets for the treatment of osteoporosis. *Osteoporos Int* 14 (Suppl. 3): 2-8.
- Shaffer RA (2001) Incidence and prevalence of stress fractures in military and athletic populations. In: Burr DB & Milgrom C (eds) *Musculoskeletal fatigue and stress fractures*. CRC Press LLC, Florida p. 1-14.
- Shmoekler Reis RJ & Ebert RH (2003) Animal models for discovery and assessment of genetic determinants of osteoporosis. *Osteoporos Int* 14 (Suppl. 5): 100-106.
- Sillence DO, Senn A & Danks DM (1979) Genetic heterogeneity in osteogenesis imperfecta. *J Med Genet* 16: 101-116.
- Sillence D (1981) Osteogenesis imperfecta: an expanding panorama of variants. *Clin Orthop* 159: 11-25.
- Sillence DO, Barlow KK, Garber AP, Hall JG & Rimoin DL (1984) Osteogenesis imperfecta type II delineation of the phenotype with reference to genetic heterogeneity. *Am J Med Genet* 17: 407-423.
- Sillence DO (1988) Osteogenesis imperfecta nosology and genetics. *Ann N Y Acad Sci* 543:1-15.
- Singer A, Ben-Yehuda O, Ben-Ezra Z & Zaltman S (1990) Multiple identical stress fractures in monozygotic twins. *J Bone Jt Surg* 72: 444.
- Slayton RL, Deschenes SP & Willing MC (2000) Nonsense mutations in the COL1A1 gene preferentially reduce nuclear levels of mRNA but not hnRNA in osteogenesis imperfecta type I cell strains. *Matrix Biol* 19: 1-9.

- Sly WS, Hewett-Emmett D, Whyte MP, Yu YS & Tashian RE (1983) Carbonic anhydrase II deficiency identified as the primary defect in the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. *Proc Natl Acad Sci USA* 80: 2752-2756.
- Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB & Korach KS (1994) Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *N Engl J Med* 20: 1056-1061.
- Sommerfeldt DW & Rubin CT (2001) Biology of bone and how it orchestrates the form and function of the skeleton. *Eur Spine J* 10 (Suppl. 2): 86-95.
- Sommarin Y, Wendel M, Shen Z, Hellman U & Heinegård D (1998) Osteoadherin, a cell-binding keratan sulfate proteoglycan in bone, belongs to the family of leucine-rich repeat proteins of the extracellular matrix. *J Biol Chem* 273: 16723-16729.
- Spotila LD, Colige A, Sereda L, Constantinou-Deltas CD, Whyte MP, Riggs BL, Shaker JL, Spector TD, Hume E & Olsen N (1994) Mutation analysis of coding sequences for type I procollagen in individuals with low bone density. *J Bone Miner Res* 9: 923-932.
- Stacey A, Bateman J, Choi T, Mascara T, Cole W & Jaenisch R (1988) Perinatal lethal osteogenesis imperfecta in transgenic mice bearing an engineered mutant pro-alpha 1(I) collagen gene. *Nature* 332: 131-136.
- Steelman J & Zeitler P (2001) Osteoporosis in pediatrics. *Pediatr Rev* 22: 56-65.
- Stewart EJ & O'Reilly BF (1989) A clinical and audiological investigation of osteogenesis imperfecta. *Clin Otolaryngol* 14: 509-514.
- Stewart TL & Ralston SH (2000) Role of genetic factors in the pathogenesis of osteoporosis. *J Endocrinol* 166: 235-245.
- Superti-Furga A, Steinmann B & Perfumo F (1986) Osteoporosis-pseudoglioma or osteogenesis imperfecta? *Clin Genet* 29: 184-185.
- Sykes B, Ogilvie D, Wordsworth P, Wallis G, Mathew C, Beighton P, Nicholls A, Pope FM, Thompson E, Tsiouras P, Schwartz R, Jenssen O, Arnason A, Borresen A-L, Heiberg A, Frey D & Steinmann B (1990) Consistent linkage of dominantly inherited osteogenesis imperfecta to the type I collagen loci: COL1A1 and COL1A2. *Am J Hum Genet* 46: 293-307.
- Szpirer J, Szpirer C, Riviere M, Levan G, Marynen P, Cassiman JJ, Wiese R & DeLuca HF (1991) The Sp1 transcription factor gene (SP1) and the 1,25-dihydroxyvitamin D3 receptor gene (VDR) are colocalized on human chromosome arm 12q and rat chromosome 7. *Genomics* 11: 168-173.
- Tamai K, Semenov M, Kato Y, Spokony R, Liu C, Katsuyama Y, Hess F, Saint-Jeannet JP & He X (2000) LDL-receptor-related proteins in Wnt signal transduction. *Nature* 407: 530-535.
- Tamai K, Zeng X, Liu C, Zhang X, Harada Y, Chang Z & He X (2004) A mechanism for Wnt coreceptor activation. *Mol Cell* 13: 149-156.
- Tao C, Yu T, Garnett S, Briody J, Knight J, Woodhead H & Cowell CT (1998) Vitamin D receptor alleles predict growth and bone density in girls. *Arch Dis Child* 79: 488-494.
- Tassabehji M, Read AP, Newton VE, Harris R, Balling R, Gruss P & Strachan T (1992) Waardenburg's syndrome patients have mutations in the human homologue of the Pax-3 paired box gene. *Nature* 355: 635-636.
- Uitterlinden AG, Burger H, Huang Q, Yue F, McGuigan FE, Grant SF, Hofman A, van Leeuwen JP, Pols HA & Ralston SH (1998) Relation of alleles of the collagen type I-alpha-1 gene to bone density and the risk of osteoporotic fractures in postmenopausal women. *New Eng J Med* 338: 1016-1021.
- Uitterlinden AG, Fang Y, Bergink AP, van Meurs JB, van Leeuwen HP & Pols HA (2002) The role of vitamin D receptor gene polymorphisms in bone biology. *Mol Cell Endocrinol* 197: 15-21.
- Van Wesenbeeck L, Cleiren E, Gram J, Beals RK, Benichou O, Scopelliti D, Key L, Renton T, Bartels C, Gong Y, Warman ML, De Vernejoul MC, Bollerslev J & Van Hul W (2003) Six Novel Missense Mutations in the LDL Receptor-Related Protein 5 (LRP5) Gene in Different Conditions with an Increased Bone Density. *Am J Hum Genet* 72: 763-771.
- Wallis GA, Sykes B, Byers PH, Mathew CG, Viljoen D & Beighton P (1993) Osteogenesis imperfecta type III: Mutations in the type I collagen structural genes, COL1A1 and COL1A2, are not necessarily responsible. *J Med Genet* 30: 492-496.

- Wehrli M, Dougan ST, Caldwell K, O'Keefe L, Schwartz S, Vaizel-Ohayon D, Schejter E, Tomlinson A & DiNardo S (2000) Arrow encodes an LDL-receptor-related protein essential for Wingless signalling. *Nature* 407: 527-530.
- Wendel M, Sommarin Y & Heinegard D (1998) Bone matrix proteins: isolation and characterization of a novel cell-binding keratan sulfate proteoglycan (osteoadherin) from bovine bone. *J Cell Biol* 141: 839-847.
- Werle E, Schneider C, Renner M, Volker M & Fiehn W (1994) Convenient single-step, one tube purification of PCR products for direct sequencing. *Nucleic Acids Res* 22: 4354-4355.
- de Wet W, Bernard M, Benson-Chanda V, Chu ML, Dickson L, Weil D & Ramirez F (1987) Organization of the human pro-alpha 2(I) collagen gene. *J Biol Chem* 262: 16032-16036.
- Whitfield GK, Selznick SH, Haussler CA, Hsieh J-C, Galligan MA, Jurutka PW, Thompson PD, Lee SM, Zerwekh JE & Haussler MR (1996) Vitamin D receptors from patients with resistance to 1,25-dihydroxyvitamin D<sub>3</sub>: Point mutations confer reduced transactivation in response to ligand and impaired interaction with the retinoid X receptor heterodimeric partner. *Mol Endocrinol* 10: 1617-1631.
- Whyte MP, Murphy WA, Fallon MD, Sly WS, Teitelbaum SL, McAlister WH & Avioli LV (1980) Osteopetrosis, renal tubular acidosis and basal ganglia calcification in three sisters. *Am J Med* 69: 64-74.
- Whyte MP (2002) Osteopetrosis. In: Royce PM & Steinmann B (eds). *Connective Tissue and Its Heritable Disorders: Molecular, Genetic, and Medical Aspects*. Wiley-Liss, New York p. 789-807.
- Whyte MP, Obrecht SE, Finnegan PM, Jones JL, Podgornik MN, McAlister WH & Mumm S (2002) Osteoprotegerin deficiency and juvenile Paget's disease. *New Eng J Med* 347: 175-184.
- Willing MC, Pruchno CJ, Atkinson M & Byers PH (1992) Osteogenesis imperfecta type I is commonly due to a COL1A1 null allele of type I collagen. *Am J Hum Genet* 51: 508-515.
- Willing MC, Deschenes SP, Scott DA, Byers PH, Slayton RL, Pitts SH, Arikat H & Roberts EJ (1994) Osteogenesis imperfecta Type I: molecular heterogeneity for COL1A1 null alleles of type I collagen. *Am J Hum Genet* 55: 638-647.
- Willing MC, Deschenes SP, Slayton RL & Roberts EJ (1996) Premature chain termination is a unifying mechanism for COL1A1 null alleles in osteogenesis imperfecta type I cell strains. *Am J Hum Genet* 59: 799-809.
- Wolf U (1997) Identical mutations and phenotypic variation. *Hum Genet* 100: 305-321.
- Yoo TJ, Chiang TM, Dixit S, Sudo N, Takeda T, Ishibe T & Seyer J (1988) Collagen components of bovine fetal and guinea pig cochlear bone and human stapes. *Ann Otol Rhinol Laryngol* 97: 318-321.
- Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T & Kato S (1997) Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nature Genet* 16: 391-396.
- Young ID, Thompson EM, Hall CM & Pembrey ME (1987) Osteogenesis imperfecta type IIA: evidence for dominant inheritance. *J Med Genet* 24: 386-389.