MECHANISMS IN ENDOCRINOLOGY

Genetics of human bone formation

Eveline Boudin and Wim Van Hul
Center of Medical Genetics, University of Antwerp, Antwerp, Belgium

Abstract
Throughout life, bone is continuously remodelled to be able to fulfil its multiple functions. The importance of strictly regulating the bone remodelling process, which is defined by the sequential actions of osteoclasts and osteoblasts, is shown by a variety of disorders with abnormalities in bone mass and strength. The best known and most common example of such a disorder is osteoporosis, which is marked by a decreased bone mass and strength that consequently results in an increased fracture risk. As osteoporosis is a serious health problem, a large number of studies focus on elucidating the aetiology of the disease as well as on the identification of novel therapeutic targets for the treatment of osteoporotic patients. These studies have demonstrated that a large amount of variation in bone mass and strength is often influenced by genetic variation in genes encoding important regulators of bone homeostasis. Throughout the years, studies into the genetic causes of osteoporosis as well as several rare monogenic disorders with abnormal high or low bone mass and strength have largely increased the knowledge on regulatory pathways important for bone resorption and formation. This review gives an overview of genes and pathways that are important for the regulation of bone formation and that are identified through their involvement in monogenic and complex disorders with abnormal bone mass. Furthermore, novel bone-forming strategies for the treatment of osteoporosis that resulted from these discoveries, such as antibodies against sclerostin, are discussed as well.

Introduction
The human skeleton has several functions such as support and protection of soft tissue. To maintain bone strength, skeletal bones are subjected to continuous bone remodelling. During the bone remodelling process, the activity of the bone-resorbing osteoclasts and bone-forming osteoblasts is well balanced and strictly regulated to maintain bone mass and bone strength.

The osteocytes, differentiated from osteoblasts and located in the mineralised bone have, together with several signalling pathways, an important role in the regulation of bone remodelling (1, 2, 3). Disturbance of the balance between bone resorption and bone formation can result in pathological abnormalities in bone mineral density (BMD).

Invited Author’s profile
Wim Van Hul, Msc, PhD, is full-time Professor of Molecular Biology and Genetics at the University of Antwerp, Belgium. He is in charge of a research team studying genetic factors underlying skeletal disorders and obesity. They were successful in identifying and characterising several disease-causing genes including the SOST gene encoding the sclerostin protein.
Osteoporosis is the most prevalent metabolic bone disease and is marked by a decreased BMD that results in increased fracture risk. It has, due to its high prevalence, hospitalisation, morbidity and mortality, a high socio-economic burden, which makes prevention, diagnosis and treatment an important research topic (4). BMD is a skeletal trait that is widely used as a surrogate phenotype for several common (for example, osteoporosis) and rare bone disorders. It is reported as a T- or Z-score with the T-score representing the number of standard deviations above or below the average peak bone mass of young adults, whereas the Z-score represents the number of standard deviations above or below the average BMD of an individual of the same age (5, 6). A T-score between −1 and 1 is considered normal, whereas a score below or equal to −2.5 indicates osteoporosis (7).

Variation in BMD is caused by a combination of environmental and genetic factors. Heritability studies have shown that the genetic impact on BMD can vary between different skeletal sites, but overall, 50–85% of the variation in BMD is due to genetic factors (8, 9, 10, 11, 12). In most cases, the genetic impact is determined by genetic variation in a large amount of genes with small effect sizes. In a minority of cases, one mutation in one gene has a major effect on bone mass or bone strength. Although this is only true in a small number of cases, identification of these genes largely increased the knowledge of pathways that regulate bone homeostasis (4, 8, 13). Furthermore, genome-wide association studies (GWAS) that aim to identify genes and variants involved in the regulation of BMD in the general population have confirmed the importance of the same pathways in the regulation of bone homeostasis (14, 15, 16).

The list with monogenic skeletal disorders is extensive and is reviewed in 2015 by Bonafe in the nosology of skeletal disorders that includes 364 different genes involved in the aetiology of 436 different skeletal disorders (17). In this review, we will only focus on a small subset of genes and pathways that are known to be involved in the regulation of bone formation and that are identified either by studying human monogenic bone disorders with high or low BMD or by performing genome-wide association studies in large cohorts in an attempt to explain the complex genetic architecture of BMD.

**Monogenic bone disorders**

As mentioned in the introduction, in a small number of cases mutations in one gene have a major effect on bone mass and strength. These so-called monogenic bone disorders are usually rare disorders with a clear mode of inheritance. Although the prevalence of these disorders is rare, a large number of different monogenic disorders have been described. Depending on the genetic defect, disorders with an increased fracture risk due to low BMD or impaired microarchitecture of the bone tissue and disorders with increased bone mass can be distinguished. Based on their function, we can classify the majority of these disease-causing genes in one of three major signalling pathways regulating bone formation, namely collagen 1 biosynthesis, WNT signalling and TGFβ signalling. Finally, for some other disease-causing genes, the function in bone formation is still unclear or independent of the previously mentioned signalling pathways.

**Collagen type 1 metabolism**

Collagen, more specifically collagen type 1, is the most important and abundant organic component of the extracellular bone matrix that provides the bone strength and flexibility. Collagen type 1 in bone consists of three polypeptide chains (α-chains) that form a triple-helix structure (18, 19). Mutations in one of the genes responsible for collagen type 1 synthesis and assembly can consequently be the cause of brittle bone disease or alternatively called osteogenesis imperfecta (OI). OI is the collective name of a clinically and genetically heterogeneous group of connective tissue disorders. In addition to low bone mass and fractures, patients with OI often suffer from other connective tissue symptoms such as blue sclerae and dentinogenesis imperfecta (19, 20, 21). Radiographically, five different OI types can be distinguished (OI type I–V). OI type II is the most severe form and often results in perinatal death, whereas OI types I and IV are the mildest non-deforming types (17, 21). Type V is the most recently identified type of OI that is diagnosed in less than 5% of the OI cases (22, 23). In around 90% of the osteogenesis imperfecta cases, autosomal dominant (AD) mutations in COL1A1 and COL1A2 are identified. Depending on the nature and localisation of the mutation, different types of OI (types I–IV) can be caused by mutations in these genes (Table 1) (24). The remaining 10% of the OI type I–IV cases are autosomal recessive (AR), and throughout the years, mutations in a total of 14 different genes are identified (4, 8, 25). The majority of these genes, namely CRTAP, LEPREI1, PLOD2, CREB3L1, PPIB, SERPINH1, SERPINF1, FKBPI0, TMEM38B, MBTPS2, SPARC and BMP1 are directly

---

**Table 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL1A1</td>
<td>Collagen type 1 alpha 1 chain</td>
</tr>
<tr>
<td>COL1A2</td>
<td>Collagen type 1 alpha 2 chain</td>
</tr>
<tr>
<td>CRTAP</td>
<td>Chondroitin sulfate proteoglycan 6 type A protein</td>
</tr>
<tr>
<td>LEPREI1</td>
<td>Lens epithelium-derived growth factor</td>
</tr>
<tr>
<td>PLOD2</td>
<td>Procollagen-processing endopeptidase</td>
</tr>
<tr>
<td>CREB3L1</td>
<td>CAMP-responsive element-binding protein 3-like 1</td>
</tr>
<tr>
<td>PPIB</td>
<td>Pseudopodium-like protein 1</td>
</tr>
<tr>
<td>SERPINH1</td>
<td>Serine protease inhibitor, clade H (11), member 1</td>
</tr>
<tr>
<td>SERPINF1</td>
<td>Serpine 1</td>
</tr>
<tr>
<td>FKBPI0</td>
<td>Fibrillin-binding protein 10</td>
</tr>
<tr>
<td>TMEM38B</td>
<td>Transmembrane protein 38B</td>
</tr>
<tr>
<td>MBTPS2</td>
<td>Membrane traffic protein, sortase B-related</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein, acidic and rich in cysteine</td>
</tr>
<tr>
<td>BMP1</td>
<td>Bone morphogenetic protein 1</td>
</tr>
</tbody>
</table>

---
or indirectly involved in the collagen 1 synthesis and assembly (Fig. 1 and Table 1) (19, 20, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38). Similar to what is seen in patients with mutations in COL1A1 and COL1A2, in some of the genes, different mutations gave rise to different forms of OI. Furthermore, in literature, other disorders with decreased BMD and decreased bone strength like Bruck syndrome and Cole-Carpenter disease are reported to be caused by genes involved in collagen 1 biosynthesis (4, 8). These disorders are referred to as OI-like syndromes, and in case of Bruck syndrome, mutations in two known OI genes FKBP10 and PLOD2, are reported to be causative (Table 1). Patients with Bruck syndrome have in addition to the OI phenotype also contractures of the large joints and pterygia (21). Cole-Carpenter is characterised by bone deformities that are reminiscent for OI as well as by distinct facial features and ocular proptosis with orbital craniosynostosis (39). The disease was already described in 1987, but recently, whole-exome sequencing (WES) resulted in the identification of P4HB and SEC24D, both involved in collagen 1 biosynthesis, as disease-causing genes for AD and AR forms of Cole-Carpenter disease respectively (Fig. 1 and Table 1) (40, 41). These findings confirm the importance of collagen 1 metabolism in defining bone mass and bone strength.

In addition to the genes involved in collagen 1 biosynthesis, mutations in genes involved in other pathways important for human bone formation are reported to cause OI. For example, mutations in WNT1, a ligand of the WNT signalling pathway and mutations in Sp7, encoding osterix, an important regulator of mesenchymal stem cell differentiation can cause OI types III and IV (Table 1) (42, 43, 45, 46). Finally, OI type V is caused by mutations in the IFITM5 gene of which the function is still largely unknown (23).

### WNT signalling

WNT signalling is an important pathway known for several decades and involved in a large variety of cellular functions. In the context of bone formation, WNT proteins act as ligands for Wnt receptor complexes, which induces downstream signals that ultimately regulates osteoblast differentiation and/or survival (47). An important subclass of WNT proteins is the WNT3 family, consisting of WNT1, WNT2, WNT2B, WNT3, WNT3A and WNT10A, all of which are expressed in bone-forming tissue (48). In particular, WNT1 has been extensively studied with respect to its role in bone formation. WNT1 is expressed in osteoblasts, where it plays a crucial role in the differentiation of mesenchymal stem cells into osteoblasts (49). Moreover, WNT1 is also important for the survival of fully differentiated osteoblasts (50). In addition, WNT1 has been shown to induce the expression of the osteoblast marker Sox9 (51), which is required for the maintenance of the osteoblast population (52). These findings indicate that WNT1 is a key player in the osteoblast differentiation process and that mutations in WNT1 can lead to bone formation defects.

### Table 1 Overview of disease-causing genes involved in human bone formation for monogenic bone disorders marked by high or low bone mass.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Bone mass</th>
<th>Gene</th>
<th>Disorder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen type 1 metabolism</td>
<td>Low bone mass</td>
<td>Col1A1, Col1A2</td>
<td>OI type I, II, III, IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CRTAP, LEPRE1</td>
<td>OI type II, III, IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPIB, BMP1</td>
<td>OI type II, III, IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FKBP10, PLOD2</td>
<td>OI type III, IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SERPINH1, CREB3L1</td>
<td>OI type III</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SERPINF1, MBTPS2</td>
<td>OI type III, IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SPARC</td>
<td>OI type IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P4HB</td>
<td>Cole-Carpenter syndrome type I, AD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SEC24D</td>
<td>Cole-Carpenter syndrome type II, AR</td>
</tr>
<tr>
<td>WNT signalling</td>
<td>Low bone mass</td>
<td>WNT1, LRPS</td>
<td>OI type II, IV; Juvenile idiopathic osteoporosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LRPS</td>
<td>Juvenile idiopathic osteoporosis; Osteoporosis pseudoglioma</td>
</tr>
<tr>
<td></td>
<td>High bone mass</td>
<td>SOST, LRP4</td>
<td>HBM phenotype, endosteal hyperostosis, Worth disease, osteosclerosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WTX</td>
<td>Sclerosteosis; Van Buchem disease, Craniodiaphyseal dysplasia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LGP5</td>
<td>Osteopetrosis; Buschke–Ollendorff syndrome</td>
</tr>
<tr>
<td>TGFβ signalling</td>
<td>High bone mass</td>
<td>TGfβ1, LEMD3</td>
<td>Camurati-Engelmann disease</td>
</tr>
<tr>
<td>Osteogenic cell differentiation</td>
<td>Low bone mass</td>
<td>Sp7</td>
<td>OI type III, IV</td>
</tr>
<tr>
<td></td>
<td>High bone mass</td>
<td>GJA1</td>
<td>Craniometaphyseal dysplasia, AR</td>
</tr>
<tr>
<td>Mineralization</td>
<td>Low bone mass</td>
<td>IFITM5</td>
<td>OI type V</td>
</tr>
<tr>
<td></td>
<td>High bone mass</td>
<td>ANKH</td>
<td>Craniometaphyseal dysplasia, AD</td>
</tr>
<tr>
<td>Unknown</td>
<td>Low bone mass</td>
<td>PLS3</td>
<td>X-linked osteoporosis</td>
</tr>
</tbody>
</table>

or indirectly involved in the collagen 1 synthesis and assembly (Fig. 1 and Table 1) (19, 20, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38). Similar to what is seen in patients with mutations in COL1A1 and COL1A2, in some of the genes, different mutations gave rise to different forms of OI. Furthermore, in literature, other disorders with decreased BMD and decreased bone strength like Bruck syndrome and Cole-Carpenter disease are reported to be caused by genes involved in collagen 1 biosynthesis (4, 8). These disorders are referred to as OI-like syndromes, and in case of Bruck syndrome, mutations in two known OI genes FKBP10 and PLOD2, are reported to be causative (Table 1). Patients with Bruck syndrome have in addition to the OI phenotype also contractures of the large joints and pterygia (21). Cole-Carpenter is characterised by bone deformities that are reminiscent for OI as well as by distinct facial features and ocular proptosis with orbital craniosynostosis (39). The disease was already described in 1987, but recently, whole-exome sequencing (WES) resulted in the identification of P4HB and SEC24D, both involved in collagen 1 biosynthesis, as disease-causing genes for AD and AR forms of Cole-Carpenter disease respectively (Fig. 1 and Table 1) (40, 41). These findings confirm the importance of collagen 1 metabolism in defining bone mass and bone strength.

In addition to the genes involved in collagen 1 biosynthesis, mutations in genes involved in other pathways important for human bone formation are reported to cause OI. For example, mutations in WNT1, a ligand of the WNT signalling pathway and mutations in Sp7, encoding osterix, an important regulator of mesenchymal stem cell differentiation can cause OI types III and IV (Table 1) (42, 43, 45, 46). Finally, OI type V is caused by mutations in the IFITM5 gene of which the function is still largely unknown (23).
processes. The pathway is activated when a WNT ligand binds a Frizzled (Fz) receptor. So far, 19 WNT ligands and 10 Fz receptors have been described and depending on the ligand, receptor and possible interactions with co-receptors, three different WNT signalling cascades, one canonical pathway or two non-canonical pathways, can be activated (47). In the beginning of this century, it became clear that especially canonical WNT signalling is one of the major pathways regulating bone formation (48, 49, 50, 51). The pathway is activated by the binding of a WNT ligand on a receptor complex formed by an Fz receptor and an LRP5/6 co-receptor (Fig. 1). Activation of the pathway results intracellularly in the stabilisation of β-catenin that can translocate to the nucleus where it can modulate the transcription of target genes. The canonical WNT signalling pathway can be distinguished from the non-canonical pathways based on the use of LRP5/6 as co-receptor and its intracellular signalling via β-catenin (Fig. 1) (47, 52). A great amount of evidence for a role of this pathway in the regulation of bone formation was found through the identification of mutations in disorders characterised by an extreme high or low bone mass (Table 1) (43). In addition to mutations in WNT1, juvenile osteoporosis can also be caused by heterozygous loss-of-function mutations in LRP5, a co-receptor of the canonical WNT signalling pathway (57). Moreover, homozygous mutations in LRP5 are shown to cause osteoporosis pseudoglioma (OPPG), another disorder with a highly reduced bone mass and increased fracture risk (Table 1) (50, 58, 59).

Further proof of the importance of LRP5 in the regulation of bone formation is found by the identification of gain of function in patients with autosomal dominantly inherited bone disorders marked by an increased bone density such as the high bone mass (HBM) phenotype, endosteal hyperostosis, Worth disease and osteosclerosis (Table 1) (51, 60, 61). All identified mutations colocalise in the first β-propeller domain of the receptor, which is shown to be important for the binding to sclerostin and members of the dickoppf (DKK) family, all inhibitors of the canonical WNT signalling pathway. Due to the HBM mutations, the binding between LRP5 and its antagonists, sclerostin and DKK1, is lost which results in increased activation of the pathway and ultimately in increased bone formation (Fig. 1) (62, 63, 64). Sclerostin, encoded by the SOST gene, is another important regulator of bone formation, which is identified through the study of monogenic bone disorders. Deletion of a 52-kb large region, located 35 kb downstream of SOST and harbouring an enhancer, is shown to cause Van Buchem disease while loss-of-function mutations in the same gene are found in
patients with sclerosteosis (48, 49). Van Buchem disease and sclerosteosis are both autosomal recessive bone disorders that are both marked by hyperostosis of the skull and tubular bones. Sclerosteosis is the more severe disorder of the two and some sclerosteosis patients deceased as a consequence of the increased intracranial pressure due to increased thickness of the skull (65). In addition, mutations in \( SOST \) were also identified in patients diagnosed with craniodiaphyseal dysplasia, a rare autosomal dominantly inherited disorder marked by generalised hyperostosis and characteristic facial dysmorphisms referred to as lion face, again confirming the importance of sclerostin in the regulation of bone formation (Fig. 1) (66). More recently, we reported that hypomorphic mutations in another player of the WNT signalling pathway, namely \( LRP4 \), cause sclerosteosis (Table 1) (53, 54). Functional studies demonstrated that \( LRP4 \) can modulate the WNT signalling pathway by facilitating the inhibitory actions of sclerostin (Fig. 1). All identified mutations in \( LRP4 \) colocalise in the third \( \beta \)-propeller domain of the receptor and result in decreased binding with sclerostin, which consequently results in increased activity of the WNT signalling pathway and increased bone formation (53, 54). Another skeletal disorder caused by mutations in a modulator of the WNT signalling pathway is Pyle’s disease (OMIM 265900), an autosomal recessive disorder that was first described in 1933 and is marked by long bones with wide and expanded trabecular metaphyses, thin cortical bone and bone fragility. Recently, homozygous truncating mutations in the gene encoding secreted Frizzled-related protein 4 (sFRP4) were shown to be disease causing in patients with Pyle’s disease (67, 68). sFRP4 can bind to WNT ligands, and in this way, it can modulate all different WNT signalling pathways. In mice lacking \( Sfrp4 \), the amounts of trabecular bone was increased and the cortical bone is unusually thin, which resembles the phenotype seen in patients with Pyle’s disease (68). Finally, extracellular modulators of the WNT signalling pathway are not alone to carry mutations. Osteopathia striata, an X-linked disorder, is caused by inactivating mutations in \( AMER1 \), alternatively called \( WTX \), an intracellular inhibitor of the pathway (Fig. 1 and Table 1) (55, 56). The disease is marked by longitudinal striations of long bones, macrocephaly, cleft palate and hearing loss in females; however, large phenotypic variation is observed due to non-random X-inactivation. In males, osteopathia striata is usually mortal in neonatal or foetal stage (56).

So, identification of above described disease-causing genes and subsequent \textit{in vitro} and \textit{in vivo} studies demonstrated that canonical WNT signalling is an important regulator of bone formation through the regulation of osteoblast proliferation and differentiation and matrix mineralisation (47). In addition to the role of WNT signalling in the regulation of bone formation, studies demonstrated that activation of the pathway influences bone resorption as well by increasing the expression of osteoprotegerin (OPG) (69). OPG inhibits osteoclastogenesis and consequently bone resorption through its binding with the RANK ligand (RANKL). In this way, it prevents the binding of RANKL to the RANK receptor and subsequently the activation of the NF-\( \kappa \)B pathway, which stimulates osteoclastogenesis (Fig. 1) (70).

**TGF\( \beta \) signalling**

A third important pathway in the regulation of bone homeostasis is the TGF\( \beta \) signalling pathway (71). In humans, mutations in some of the players of this pathway have shown to cause rare monogenic disorders with increased bone mass due to increased bone formation (Table 1). \textit{In vivo} and \textit{in vitro} studies demonstrated that the TGF\( \beta \) signalling pathway can affect both bone formation and resorption and is an important regulator of the bone remodelling process (71).

More than 15 years ago, mutations in \( TGF\beta1 \) were identified to be causative for Camurati-Engelmann disease (CED), an autosomal dominantly inherited bone disorder marked by generalised sclerosis, hyperostosis of the skull base, thickening of the cortex in the long bones, chronic bone pain and muscle weakness. The identified mutations are gain-of-function mutations and almost all mutations are located in the latency-associated protein (LAP) region of TGF\( \beta1 \) (72, 73, 74). Non-covalent linking of the LAP domain to the receptor-binding domains of active TGF\( \beta1 \) is important to keep TGF\( \beta1 \) inactive in the bone matrix. During bone resorption, active TGF\( \beta1 \) will be released from the bone matrix (Fig. 1). Due to the CED mutations, the release of active TGF\( \beta1 \) from the bone matrix is increased. Furthermore, \textit{in vitro} and \textit{in vivo} studies demonstrated that active TGF\( \beta1 \) is necessary for the migration and proliferation of osteoprogenitor cells to the bone surface after bone resorption. So, due to the CED-causing mutations in \( TGF\beta1 \), there will be an increased release of active TGF\( \beta1 \), which results ultimately in abnormal bone remodelling and increased the bone turnover (74).

Osteopoikilosis or alternatively called spotted bone disease is characterised by the occurrence of small focal sclerotic lesions in one or multiple bones (75). When the bone phenotype is accompanied by connective tissue nevi,
it is referred to as Buschke–Ollendorff syndrome (BOS) (76). Studies have shown that this autosomal dominantly inherited disorder is caused by inactivating mutations in the LEMD3 gene. LEMD3 is a nuclear membrane protein that can inhibit the TGFβ signalling pathway by binding to SMAD proteins, the downstream mediators of the pathway (Fig. 1) (76, 77). In some families, osteopoikilosis and BOS occur together with melorheostosis, a condition characterised by asymmetric lesions in the cortex of tubular bones. In addition to the bone, adjacent tissues such as skin and muscles are often also affected. This leads to the suggestion that these conditions might be allelic; however, several studies were unable to confirm this (76, 77).

**Other or unknown function**

Novel technologies have led to the identification of novel genes for monogenic bone disorders. Several of these genes are involved in the above described pathways or other not yet mentioned pathways. However, the exact working mechanism or pathway for all identified genes is not known.

In the section on osteogenesis imperfecta, we mentioned that not all of the identified genes are involved in collagen 1 biosynthesis. One of these genes is Sp7, encoding for the well-known osteoblast-specific transcription factor osterix that regulates osteoblast differentiation (Table 1). Loss of osterix function results in reduced osteoblast differentiation and bone formation (44). Another previously mentioned osteogenesis imperfecta gene is IFITM5. So far, it is the only gene known to cause OI type 5 (Table 1) (23). In vitro studies have shown that IFITM5 is expressed in the osteoblasts during the early mineralisation phase. In vitro data suggest that IFITM5 has a role during mineralisation, but no serious bone phenotype could be found in an ifitm5-knockout mouse model (23). Based on these data, more research is needed to further elucidate the role of IFITM5 in the regulation of bone formation.

A few years ago, mutations in PLS3 were identified using exome sequencing in a family with X-linked osteoporosis (Table 1) (78, 79). The role of PLS3 in bone remodelling is still unknown but based on its capacity to bind actin and the expression of PLS3 in the osteocytes, a role in the mechanosensing by the osteocytes is suggested (79, 80). As an adaption to mechanical loading, the osteocyte induces increased bone formation for example by suppressing SOST expression. In this way, mutations in PLS3 could explain the osteoporosis phenotype in the patients.

In addition to the above described disorders with a decreased bone mass, there are also monogenic bone disorders with increased bone mass due to defects in osteoblasts that are caused by genes with rather unknown function in bone metabolism. Perhaps the most severe disorder is Raine syndrome caused by loss-of-function mutations in FAM20C (81). Raine syndrome is an autosomal recessive disorder that in most cases is fatal in the first weeks of life (82). The disease is characterised by generalised osteosclerosis and typical facial features such as midface hypoplasia, hypoplastic nose, exophthalmos and low set ears. In surviving patients, the disease is caused by hypomorphic mutations in FAM20C and in addition to the above described features, surviving patients often also have amelogenesis imperfecta and mild hypophosphatemia (81, 83, 84). The disease-causing gene, FAM20C, alternatively called dentin matrix protein 4, is highly expressed in odontoblasts, ameloblasts, cementoblasts, osteoblasts and osteocytes (85) and encodes for a Golgi casein kinase that is involved in the phosphorylation of secretory calcium-binding phosphoprotein members such as for example, dentin matrix protein 1 (DMP1), osteopontin (OPN), matrix extracellular phosphoglycoprotein (MEPE) and bone sialoprotein (BSP). The contribution of these proteins to mineralisation is complex; however, it has been reported that abnormal phosphorylation of these proteins is involved in the aberrant mineralisation observed in Raine syndrome (Fig. 1) (86, 87, 88).

Finally, ANKH and GJA1 are reported to cause respectively autosomal dominant and recessive forms of craniofacial dysplasia (CMD) (Table 1). Individuals with CMD usually have generalised sclerosis and a characteristic facial appearance. Frequently, signs of cranial nerve compression such as hearing loss, impaired vision or facial paralysis are also reported (89). The AD form is more common and milder than the AR recessive form. It is caused by loss-of-function mutations in the ANKH gene, encoding a pyrophosphate transporter (90, 91). ANKH seems to have multiple functions in the bone formation process. On the one hand, it is reported to stimulate osteoblastic maturation and differentiation 92, whereas on the other hand, it is shown that it is involved in the transport of intracellular pyrophosphate to the extracellular region. Pyrophosphate is an inhibitor of the mineralisation process. Due to the mutations in ANKH, the transport of pyrophosphate out of the cell is reduced and the pyrophosphate-dependent mineralisation inhibition
is lost, which contributes to the bone overgrowth seen in CMD patients (91). The AR form of CMD is more recently reported to be caused by mutations in the gap junction protein alpha 1 gene (GJA1) (93), encoding connexin43, which is a crucial component of gap junctions and hemichannels that are important for the intracellular communication in cells. In bone, Cx43 is the most abundantly expressed connexin, and in vitro data demonstrated that Cx43 is important for the intracellular communication during osteoblast differentiation (Fig. 1) (94). Knockout of Gja1 in mice results in delayed skeletal ossification, craniofacial abnormalities and osteoblast dysfunction (95). Overall, studies have shown that connexin43 has a role in the modulation of bone modelling and bone remodelling and that it is involved in the response to hormonal and mechanical stimuli by regulating the expression of osteoanabolic and catabolic target genes. However, the exact mechanism behind this regulation needs to be further elucidated (94).

In addition, some rare genetic autoinflammatory bone syndromes are described including Majeed syndrome, deficiency of interleukin-1 receptor antagonist (DIRA) and cherubism. They are caused by the activation of the innate immune system leading to an osseous inflammatory process. The disease-causing genes for these conditions are respectively LPIN2, Interleukin-1 Receptor Antagonist ILIRN and SH3-binding protein 2 (SH3BP2) (96).

Complex bone disorders

In the introduction, we already mentioned that the above described monogenic bone disorders occur in a very small group of patients. In most individuals, BMD is defined by a complex interplay between genetic variation in many genes and environmental factors. As BMD is the most important predictor of osteoporosis, throughout the years, many studies have been performed to identify these genetic factors. Although BMD is the most important predictor of osteoporosis, clinically, the occurrence of osteoporotic fractures is more relevant and only partially correlates with BMD. Consequently, different genetic factors can contribute to BMD and osteoporotic fracture risk.

Common variants

In the last decade, several genome-wide association studies (GWASs) and meta-analyses of GWASs have been performed in populations with ever-increasing sample sizes (14, 97, 98, 99, 100, 101). Such large studies made it possible to identify associated variants in a hypothesis-free manner, which results in the identification of novel genes and previously unknown functional pathways. Throughout the years, these studies lead to the identification of a large number associated (common) variants in over 90 genes or 70 loci. For approximately half of the associated genes additional evidence that for their involvement in the regulation of bone development or metabolism is available (102). Identification of the role of the other genes in the regulation of bone metabolism will contribute to the identification of novel functional pathways. The largest GWAS published so far is the GEFOS2 study in 2012 by Estrada et al. (14). This study was able to demonstrate the association of 56 loci with BMD, which was an enormous increase compared to the previous study where 20 associated loci were identified. This increase was mainly due to the significant increase in sample size compared to the first GWAS study. In addition, the GEFOS2 study also demonstrated 14 of the 56 loci that are associated with BMD are also associated with osteoporotic fracture risk. In order to find the connection between the associated loci, pathway analysis was performed on the associated loci, which highlighted the importance of three biological pathways. First, the RANK–RANKL–OPG pathway, which is important for the differentiation and activity of osteoclasts (Fig. 1). The second connection was found between important regulators of mesenchymal stem cell differentiation, and finally, many associated genes turned out to be involved in the WNT signalling pathway (14). Identification of the WNT signalling pathway and mesenchymal stem cell differentiation as important regulators of BMD through GWASs again confirms the importance of the regulation of bone formation for BMD. Although the GEFOS2 study was able to demonstrate significant associations with many variants, only 5.8% of the genetic impact on femoral neck BMD could be explained by all variants together (14). The above presented numbers symbolise both the strength of GWAS to identify novel associated genes and variants as well as its limitation in explaining the total genetic variation of BMD. The latter implies that a lot of information remains unknown, currently referred to as the missing heritability.

In order to unravel the genetic factors contributing to the missing heritability, different approaches are undertaken. One possible strategy that can be used is increasing the sample size of the study population. However, it needs to be noted that increasing the sample size can also lead to increased heterogeneity of
the concort, reducing the chances to replicate previous findings. On the 2016 annual ASBMR meeting, the GEFOS consortium presented data of a novel effort to explain more of the heritability using the samples from the largest genome-wide genotyped cohort in the world (UK-Biobank). In this cohort, a large-scale GWAS of calcaneal eBMD (estimates bone mineral density based on quantitative ultrasound) was performed. This study identified 403 independent associated SNPs from 376 loci, which together explained around 13% of the genetic variation in eBMD (103). In the future, it will be a challenge to identify all causative SNPs and genes for these associations. Identification of these genes will indisputably result in the identification of new pathways regulating bone formation. This study provides evidence that increasing the sample size of the study population is a useful strategy to unravel at least a part of the missing heritability.

### Rare variants

In another attempt to explain the missing heritability, the focus shifts to the study of rare variants instead of common variants. The implementation of next-generation sequencing (NGS) technology in GWAS can especially be helpful in the identification of rare variants as GWAS studies only focus cover common variants. So far, only a few whole-genome sequencing (WGS) association studies focusing on BMD or osteoporotic fracture risk have been published. The first study was performed by Styrkarsdottir et al. (104) and demonstrated that a rare nonsense variant in the Leucine-Rich Repeat Containing G Protein-Coupled Receptor 4 (LGR4) gene is strongly associated with low BMD among other phenotypes in the Icelandic population with large effect sizes (OR<sub>osteoporosis</sub>: 3.27 and OR<sub>fractures</sub>: 3.12) compared to previous studies (14, 104). The associated bone and other phenotypes are similar to phenotype reported in the Lgr4-knockout mice (104, 105). In literature, it is suggested that LGR4 can regulate bone homeostasis through the regulation of both bone formation and bone resorption. Although, LGR4 is reported to be a receptor for R-spondins, which can modulate WNT signalling activity, in vitro and in vivo studies in knockout mice demonstrated that Lgr4 regulates osteoblast differentiation and mineralisation via another regulatory mechanism namely CAMP-PKA-Aft4 signalling (105). In addition, a recent study reported that Lgr4 negatively regulates osteoclast differentiation and bone resorption by interacting with RANKL (106).

As previously mentioned, RANKL–RANK interaction is important for the activation of osteoclastogenesis and bone resorption. Consequently, the reduced bone mass seen in the presence of the nonsense mutation in LGR4 and in the Lgr4-knockout mouse can not only be explained by a delay in osteoblast differentiation and mineralisation but also by an increased bone resorption (105, 106). Investigation in other populations suggested that the variant is specific for the Icelandic population. So, the role of genetic variation in LGR4 in other populations than the Icelandic population remains unclear and needs to be further investigated (104). The second study combined both data from whole genome and whole exome sequencing to impute variants that are missing in previous GWAS (107). Using this strategy, the authors identified a low-frequency non-coding variant (rs11692564, MAF 1.7%) 53 kb downstream of engrailed homeobox-1 (EN1) that is associated with both BMD (BMD<sub> lumbar spine</sub>; β=0.22) and fracture risk (OR: 0.85) (107). Although, the effect size of this variant is small compared to the effect of the variant in LGR4 described previously, it is much bigger than the effect of common variants (14, 107). Furthermore, more recently, the association of rs11692564 with BMD was replicated in a population of children and adolescents (108). EN1 expression is found in cells from osteogenic lineages and is shown to be involved in the WNT signalling pathway by interacting with the WNT signalling antagonist Dkk1. Studies in self-deleted EN1 (sdEN1) conditional mutant mice show a lower trabecular and cortical bone mass, which is suggested to be the result of increased osteoclastic activity driven by the deletion of EN1 in osteogenic cells (107). In addition, in an Icelandic population, an additional variant in the EN1 gene, rs115242848, is shown to be associated with LS and hip BMD and osteoporotic fracture by a WGS association study. Furthermore, rare variants in genes previously shown to be associated with BMD, namely SOST, AXIN1 and COLIA2, are also reported to be associated with BMD in the Icelandic population (109, 110). Moreover, the identified rare variants in SOST and COLIA2 are also associated with fracture risk. Finally, in the same population, Styrkarsdottir et al. also demonstrated that a rare variant in the PTCH1 gene is associated with BMD and fracture risk (109). PTCH1, encodes the PTCH1 receptor, which is involved in the hedgehog pathway, a pathway previously shown to be involved in the regulation osteoblastogenesis and chondrocyte differentiation (111).

Previously described studies demonstrate that WGS can be a useful technique for the identification of rare variants associated with BMD. Typically, the effect size of
these rare variants will be larger compared to the effect of common variants. However, to have enough power to identify these rare variants, very large study populations are needed. Furthermore, rare variants alone can probably not explain all the missing heritability.

**Other genetic variation**

In addition to the effect of common and rare variants on bone mass, the effect of other genetic variation such as copy number variation (CNV), DNA methylation, microRNAs (miRNAs) and long non-coding RNAs can contribute to the variation in bone mass. Recently, research into the effect of other genetic variation on bone mass and on the development of osteoporosis gained more interest.

The association of CNVs with bone mass and osteoporosis is only studied by a few GWAS. These studies reported that VSP13B, UGT2B17 and an intergenic region within the 6p25.1 locus and 20q13.12 are associated with bone mass, osteoporosis or osteoporotic fractures (112, 113, 114, 115, 116, 117).

In addition to the effect of CNVs on bone mass and osteoporosis, studies also focus on the effect of so-called epigenetic changes, miRNAs and DNA methylation, on the variation in bone mass and osteoporosis risk. Epigenetics refers to stable and heritable changes in phenotype or gene expression attributable to mechanisms other than changes in the underlying DNA sequence. These mechanisms are dependent on interactions between the environment and the genome (4, 118). MiRNAs are small non-coding RNAs that are incorporated into the RNA-induced silencing complex (RISC) and that can by binding to messenger RNA (mRNA) modulate the expression of target genes. Studies have shown that most miRNAs are able to bind and modulate several mRNAs, and most mRNAs can be targeted by several miRNAs. So far, numerous miRNA have been reported to regulate osteogenesis and osteoporosis risk (119, 120).

DNA methylation is another epigenetic mechanism that is studied for its role in the regulation of bone mass. It is a reversible modification of a cytosine residue located 5’ to a guanosine residue (CpG). DNA methylation that primarily represses gene expression by either inhibiting or facilitating binding between proteins and DNA. The role of DNA methylation in the regulation has been shown by several studies. For example, it has been shown that differentiation of osteoblasts to osteocytes is accompanied by decreased methylation of the SOST promoter, which facilitates osteocyte-specific expression of SOST (121). In addition, in osteoporosis patients, increased methylation of the SOST promoter and consequently a decreased level of sclerostin in the serum of the patients was reported (122). Not only the WNT signalling pathway but also other pathways know to be important in the regulation of bone metabolism such as the RANGL–RANK–osteoprotegerin pathway are shown to be modulated by DNA methylation (123). It is clear that in addition to common and rare variants that are mostly studied, other genetic variation also contributes to the regulation of bone formation; however, more studies are needed to further elucidate and identify these factors.

**Therapeutic opportunities**

As mentioned, osteoporosis is a common disease marked by reduced bone mass and increased fracture risk. At the moment, the treatment of osteoporosis is mainly focused on reducing bone loss. However, this is not always sufficient to prevent fractures on the long term. Using antiresorptive treatments such as bisphosphonates or teriparatide, the bone remodelling process will be inhibited and osteoblasts will not be stimulated to produce new bone. During the past years, genetic studies highlighted a large amount of genes and pathways that are important for the regulation of human bone formation. Throughout the years, studies have shown that these pathways, especially the Wnt signalling pathway, are promising targets for the treatment of osteoporosis.

So far the most important and best studied target is sclerostin. Sclerostin inhibits the WNT signalling pathway by binding to LRP5 or LRP6 and loss-of-function mutations in the SOST gene, encoding sclerostin, result in increased bone formation in sclerosteosis and Van Buchem disease patients (47). Moreover, expression of sclerostin is mainly limited to osteogenic cells. Based on these findings, several companies developed sclerostin antibodies for the treatment of osteoporosis. Most advanced are the studies of Romosozumab (Amgen and UCB) of which phase III clinical trial results are recently published (124). This study compared one-year Romosozumab treatment followed by one-year Denosumab treatment with one-year placebo treatment followed by one-year Denosumab treatment were started in post-menopausal women. The results of this study demonstrated that one-year Romosozumab treatment resulted in decreased risk of both vertebral and clinical fractures (124). Although, the results look promising, the clinical study and sclerostin antibody treatment also have some limitations as described by
S Papapoulos recently (125). The patients with severe disease (T-score \(<-3.5\)) are excluded from the study, although bone-forming therapies are designed primarily for these patients. Furthermore, phase II and III clinical trials demonstrated that continuous stimulation of bone formation through the inhibition of sclerostin is unlikely after one year (124, 126). In addition, phase III clinical trials demonstrated that the effect of Romosozumab on non-vertebral fractures is influenced by the geographical region. The study demonstrated that patients recruited from Latin America have a lower incidence of non-vertebral fractures, and only when this ethnic group is excluded from the analysis, significant results could be observed for non-vertebral fractures. Finally, in Romosozumab-treated patients, injection site reactions were more common compared to the placebo group. Furthermore, in this group, one atypical femoral fracture and two cases of osteonecrosis of the jaw were reported (124). Although these side effects are only reported in a very small number of patients and there were some confounding factors that probably contributed to the event in these patients, these findings raise some questions that need to be elucidated in the future (125).

In addition to sclerostin another modulator of the WNT signalling pathway, DKK1, is currently studied as an interesting therapeutic target (Fig. 1). DKK1 is one of the associated genes from the GWAS. Furthermore, heterozygous deletion of Dkk1 in mice results in an increased bone mass and in mice and in patients lacking sclerostin, DKK1 expression is upregulated (127, 128, 129). Based on these data and its role in the WNT signalling pathway, DKK1 was suggested to be an interesting therapeutic target. DKK1 antibodies (BHQ880, RH2-18) are studied in animal models for osteoporosis, multiple myeloma and rheumatoid arthritis (130, 131, 132, 133). Treatment of multiple myeloma with BHQ880 seems most promising and clinical trials are currently ongoing (134). Finally, another interesting target for bone-forming therapy is LRP4. As previously mentioned, LRP4 is, as a binding partner of sclerostin, also involved in the regulation of canonical WNT signalling activity and bone formation (47). Although LRP4 is more widely expressed than sclerostin, we and others demonstrated that the central cavity of the third \(\beta\)-propeller domain of the receptor is important for its role in bone formation (53, 54). Furthermore, deletion of Lrp4 only in osteoblasts or osteocytes resulted in mice in increased bone mass due to increased bone formation and decreased bone resorption (128). So, based on these findings, it seems that LRP4 can be another interesting target for the treatment of osteoporosis through bone formation; however, additional studies will be needed to confirm this.

Finally, the increased knowledge on the genetic cause of monogenic and complex bone disorders leads to novel opportunities for the treatment, prevention and diagnosis of skeletal disorders. In case of monogenic disorders, the implementation of next-generation sequencing in diagnostics will help clinicians with the diagnosis of the patients. With regard to polygenic disorders, the implementation of genetic testing is more complex. As discussed in the section on complex bone disorders, many variants with small effect sizes contribute to the variation in bone mass and fracture risk and consequently also to the risk of osteoporosis. Although already many variants have been identified through the years, they only explain a small fraction of the variation in bone mass and only have a small predictive value.

**Conclusion**

The bone remodelling process is a complex interplay between bone resorption and bone formation. Throughout the years, genetic studies largely contributed to the current knowledge on pathways regulating bone remodelling and offered several possible therapeutic targets for the treatment of osteoporosis and other bone disorders. As increasing bone formation is currently seen as the most promising strategy in the therapy of osteoporosis, we focused in this review on genetic evidence for pathways or genes involved in this process. Currently, the WNT signalling pathway is extensively studied as a possible therapeutic target, but identification of novel genes or pathways in the future will increase the insights in the regulation of bone remodelling and will definitively offer novel therapeutic targets.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality in this review.

**Funding**

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

**Acknowledgements**

The research was supported by grants of the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO grant G019712N and G031915N) and the European Community’s Seventh Framework Programme (FP7/2007–2013) under grant agreement No. 602300 (SYBIL). E B holds a postdoctoral grant with the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO personal grant 12A3814N).
Segregation analysis and variance components analysis of bone control of bone mass. (doi:10.1001/jama.286.22.2815)

Osteoporosis Risk Assessment.


Identification of a frameshift mutation in Osterix in a patient with progressive recessive osteogenesis imperfecta.


Genetics of human bone formation


64 Semenov MV & He X. LRP5 mutations linked to high bone mass diseases cause reduced LRPS binding and inhibition by SOST. *Journal of Biological Chemistry* 2006 281 38276–38284. (doi:10.1074/jbc.M609509200)


70 Boyce BF & Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Archives of Biochemistry and Biophysics* 2008 473 139–146. (doi:10.1016/j.abb.2008.03.018)

71 Wu M, Chen G & Li YP. TGF-beta and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. *Bone Research* 2016 4 16099. (doi:10.1038/boneres.2016.9)


88 www.eje-online.org
mouse progressive ankylosis gene, result in craniofacial abnormalities, and osteoblast dysfunction. Nature Genetics 2000 28 37–41. (doi:10.1038/ng0501-37)


106 Lu et al. LGR4A is a receptor for RANKL and negatively regulates osteoclast differentiation and bone resorption. Nature Medicine 2016 22 539–546. (doi:10.1038/nm.4076)


