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Genetic and functional study of LRP4 and the R-spondins in bone formation.

Genetisch en functioneel onderzoek naar de rol van LRP4 en R-spondins in de botvorming.

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SUMMARY

Osteoporosis is the most common disease affecting human bone, characterized by decreased bone mineral density (BMD) and significantly increased propensity to fractures. Currently, one in three women and one in five men over the age of 50 are expected to suffer an osteoporotic fracture, cumulating to nearly 9 million cases a year worldwide. In our aging society these numbers are only expected to rise, gradually increasing the already substantial **socioeconomic burden** of this disease.

A great majority of currently available osteoporosis treatments focus on lowering bone resorption rates, thus preventing further bone loss. However as bone resorption remains tightly linked to bone formation, all these drugs ultimately lead to a decrease in production of new bone, among other side-effects. In light of these facts, major scientific effort was devoted to the search of novel **anabolic treatment** targets, stimulating bone formation and reversing the bone loss that already occurred.

Over the years, the studies of **rare bone disorders** have contributed greatly to our understanding of skeletal biology and provided numerous contributions towards the treatment of the most common diseases affecting human bone. These contributions are discussed in detail in the first chapter of this thesis.

In particular, the identification of point mutations in the ***LRP5*** gene in high bone mass phenotype patients and in osteoporosis-pseudoglioma syndrome highlighted the vital importance of canonical **Wnt signaling** in bone health for the first time. To date, many genes involved in this pathway have been linked to the skeletal system. Recently, we and others identified the low density lipoprotein receptor-related protein 4 (**LRP4**) as a binding partner for sclerostin, a potent inhibitor of this pathway. All contributions of Wnt signaling to bone biology are reviewed in detail in the second chapter of this thesis.

The aim of this thesis was to further investigate the genes involved in the pathogenesis of different osteochondrodysplasias to **provide novel insights into the biology of human bone** and to **identify potential targets for the development of novel treatments** of common bone diseases.

In the third chapter of this thesis, the involvement of Wnt signaling coreceptor- LRP4 in bone biology is studied. We identify a **novel mutation** in the gene encoding this protein as causative for **sclerosteosis** in a Portuguese patient. The patient presented with marked sclerosis of the skull and long bones and bilateral syndactyly. The novel mutation is functionally evaluated and decreased sensitivity towards sclerostin dependent inhibition of Wnt signaling is shown in dual luciferase reporter assays. Moreover, we evaluated the effect of several previously described mutations in this gene to present a genotype-phenotype correlation for different classes of *LRP4* mutations. We show that mutations underlying **Cenani-Lenz syndrome** compromise the protein action by disrupting its localization, while sclerosteosis causing mutations selectively impair sclerostin binding without affecting the membrane trafficking of the receptor. To add to this, we show that patients suffering from sclerosteosis due to *LRP4* mutations present with **highly elevated levels of circulating sclerostin**, an observation supported by recent data coming from mouse models of LRP4 deficiency. In conclusion, we provide the first evidence of LRP4 involvement in skeletal retention of sclerostin in human bone.

In the fourth chapter of the thesis, we continue the search for novel players involved in the maintenance of bone homeostasis by investigating a group of canonical Wnt signaling modulators- the **R-spondins**. We evaluate the **expression profile** of all *RSPO* genes in differentiating osteoblasts with the use of murine KS483 cell line. Based on these results we prioritized *RSPO1*, *2* and *3* for further genetic testing. To investigate their potential role in the pathogenesis of craniotubular hyperostosis, we performed a **mutation analysis** in a population of high bone mass patients in search of rare variants underlying the phenotype. Unfortunately, we were unable to identify any possibly pathogenic variants in the genes leading us to the conclusion that mutations in the genes encoding R-spondins are not a common cause of craniotubular hyperostosis. Moreover, we evaluate the **association** between common variation in the R-spondin genes and BMD in stratified subpopulations of the Odense Androgen Study (OAS). R-spondin 3 was recently associated with bone parameters in large-scale genome-wide association studies (GWAS). We perform our **candidate association study** to investigate the GWAS findings with increased resolution and to potentially identify the causal variant. This approach recently allowed us to identify a functionally relevant, common variant in the *WNT16* gene. Unfortunately, no significant associations were detected indicating that, despite the major importance of R-spondins in the development of the skeletal system, the common variations in these genes do not contribute greatly to the genetic determination of BMD in healthy subjects. A nonsynonymous variant identified in a single individual from the low BMD subpopulation was functionally tested but showed no impact on protein activity in reporter assays. Despite these results, our data do not deteriorate the potential utility of R-spondins as drug targets in future osteoporosis treatments.

Lastly, in the fifth chapter of this thesis we investigate a Danish family diagnosed with **facioaudiosymphalangism syndrome**, another rare osteochondrodysplasia characterized by proximal symphalangism, typical facies, hyperopia, joint malformations and hearing loss. We identified a novel mutation in the gene encoding noggin (***NOG***), a secreted inhibitor of the bone morphogenic proteins (BMPs) signaling pathway. With our findings we provide novel insights into the genotype-phenotype correlation in patients suffering from *NOG*-related-symphalangism spectrum disorder.

Taken together, we believe that our studies provided novel insights into the pathogenesis of several osteochondrodysplasias. A genotype-phenotype correlation was elucidated at functional level for several mutations implicated in these disorders. Moreover, our findings highlight LRP4 as a very promising drug target for the development of future treatments of osteoporosis. This project provided the basis for the generation of an *in vivo* murine model of the mutation identified in our sclerosteosis patient which will likely contribute to the further elucidation of the LRP4 function in bone biology.

Osteoporose is de frequentst voorkomende botaandoening en wordt gekenmerkt door een sterk verlaagde bot mineraal densiteit (BMD) wat leidt tot een verhoogde kans op fracturen. Wereldwijd wordt geschat dat één op drie vrouwen en één op vijf mannen ouder dan 50 jaar een fractuur als gevolg van osteoporose zal krijgen, wat in totaal wereldwijd leidt tot 9 miljoen fracturen per jaar. Door de vergrijzing van de bevolking wordt verwacht dat dit aantal nog zal stijgen waardoor de **socio-economische last** die al een belangrijke impact heeft, nog zal toenemen. Het merendeel van de huidige behandelingen voor osteoporose is toegespitst op het verhinderen van verder botverlies door het afremmen van de botresorptie. Aangezien botaanmaak en botafbraak nauw met elkaar verbonden zijn, resulteert het afremmen van de botresorptie ook in verlaging van de botaanmaak waardoor de botdensiteit niet wordt hersteld. Bijgevolg wordt er veel onderzoek verricht naar nieuwe **anabole agentia** die de botaanmaak stimuleren en zo de botmassa kunnen herstellen.

Door de jaren heen, hebben studies naar de genetische oorzaak van **zeldzame botaandoeningen** bijgedragen tot de huidige kennis over de regulatie van de skeletontwikkeling en botremodelering. Deze kennis heeft al geleid tot de ontwikkeling van verschillende therapieën voor osteoporose welke worden beschreven in hoofdstuk 1. In het bijzonder, toonde de identificatie van puntmutaties in ***LRP5*** in zowel patiënten met het high bone mass fenotype, gekenmerkt door een verhoogde botdensiteit als in patiënten met osteoporosis pseudoglioma syndroom, een aandoening met een sterk verlaagde botdensiteit, aan dat de canonieke **Wnt signaaltransductie** een belangrijker rol speelt in de regulatie van de bothomeostase. Naast *LRP5,* is ondertussen voor verschillende andere genen uit deze pathway aangetoond dat ze een belangrijke regulerende rol spelen in skeletontwikkeling. De rol van deze spelers van de Wnt signaaltransductie in de regulatie van de bothomeostase is uitvoerig beschreven in hoofdstuk 2 van deze thesis.

De doelstelling van deze thesis was om de genen betrokken in de pathogenese van de verschillende osteochondrodysplasieën verder te onderzoeken om zo **nieuwe inzichten** te verkrijgen in de **regulatie van de humane botbiologie** wat kan leiden tot de identificatie van **potentiële nieuwe targets** voor de behandeling van botaandoeningen.

In het derde hoofdstuk van deze thesis, wordt het onderzoek naar de betrokkenheid van de Wnt signaaltransductie co-receptor LRP4 in de bothomeostase besproken. In een Portugese patiënt gediagnosticeerd met **sclerosteosis**, identificeerden we een **nieuwe mutatie** in dit gen. De patiënt vertoonde de typische kenmerken voor sclerosteosis, namelijk sclerose van de schedel en lange beenderen en bilaterale syndactyly. Het effect van de nieuwe mutatie werd functioneel bestudeerd en met behulp van dual luciferase reporter assays werd een verlaagde sensitiviteit voor de sclerostin afhankelijke inhibitie van de WNT pathway aangetoond. Daarenboven, onderzochten we het effect van verschillende reeds beschreven mutaties in *LRP4* voor sclerosteosis en het **Cenani-Lenz syndroom**, om een genotype-fenotype correlatie te kunnen opstellen voor verschillende types *LRP4* mutaties. Op deze manier konden we aantonen dat mutaties verantwoordelijk voor Cenani-Lenz syndroom de functie van het eiwit verstoren door het wijzigen van de lokalisatie van het eiwit terwijl mutaties die resulteren in sclerosteosis de binding met sclerostin verstoren. Tot slot, hebben we aangetoond dat in een patiënt met een

mutatie in *LRP4* het **serum sclerostin gehalte** sterk is verhoogd wat suggereert dat LRP4 noodzakelijk is voor de binding van sclerostin ter hoogte van het botoppervlak.

Hoofdstuk vier van deze thesis, bespreekt het onderzoek naar de rol van een groep modulatoren van de canonieke Wnt pathway, namelijk de **R-spondins**, in de regulatie van de bothomeostase. In eerste instantie hebben we een **expressie profiel** voor de verschillende *RSPO* genen tijdens de differentiatie van KS483 cellen, muriene mesenchymale stamcellen, naar osteoblasten opgesteld. Op basis van deze resultaten hebben we *RSPO1, 2* en *3* geselecteerd voor verder genetisch onderzoek. Om de rol van deze drie RSPOs in de pathogenese van craniotubulaire hyperostosen te onderzoeken, hebben we **mutatie analyse** uitgevoerd voor *RSPO1, 2* en *3* in een populatie patiënten met verschillende scleroserende aandoeningen. In deze cohorte konden we echter geen mutaties in één van de geselecteerde genen terugvinden. Bijgevolg kunnen we besluiten dat mutaties in *RSPO 1, 2 of 3* geen frequente oorzaak zijn van craniotubulaire hyperostose. Om de rol van de geselecteerde RSPOs in de regulatie van de bothomeostase verder na te gaan, hebben we **kandidaatgen associatiestudies** uitgevoerd in een subpopulatie van de Odense Androgen Study (OAS) populatie. Voor genetische variatie in *RSPO3* werd recent in een genoomwijde associatiestudie (GWAS) associatie aangetoond met botparameters. Op basis hiervan hebben we besloten om voor dit gen, een kandidaatgen associatiestudie met behulp van Sanger sequencing uit te voeren om met een hoge resolutie, de causale variant te kunnen opsporen. Deze strategie heeft ons recent toegelaten om een functioneel relevante variant geassocieerd met BMD in WNT16 te identificeren. Ondanks de belangrijke rol die de RSPO spelen in de ontwikkeling van het skelet, vonden we geen varianten die significant geassocieerd zijn met BMD in een populatie gezonde mannen. We identificeerden wel een zeldzame niet-synonieme variant in één individu van de subpopulatie met een lage botdensiteit. Functioneel onderzoek toonde aan dat deze variant in RSPO3 geen effect heeft op de functie van het eiwit. Ondanks deze resultaten, blijven R-spondins interessante targets voor behandeling van osteoporose in de toekomst.

Tenslotte, bespreken we in hoofdstuk vijf van deze thesis een Deense familie die werd gediagnosticeerd met het **facioaudiosymfalangisme syndroom**, een zeldzame osteochrondrodysplasie gekenmerkt door proximaal symfalangisme, een typische gezichtsuitdrukking, hyperopia, gewrichtsafwijkingen en gehoorverlies. In deze familie identificeerden we een nieuwe mutatie in het gen coderend voor noggin (*NOG*), een gesecreteerde inhibitor van de bone morphogenic proteins (BMP) signalisatie. De resultaten besproken in dit hoofdstuk, geven nieuwe inzichten in de genotype-fenotype correlatie bij patiënten die leiden aan *NOG*-gerelateerde symfalangisme.

We zijn ervan overtuigd dat onze studies hebben geleid tot nieuwe inzichten in de pathogenese van verschillende osteochondrodysplasieën. De genotype-fenotype correlatie is opgehelderd op functioneel niveau voor verschillende mutaties betrokken bij deze aandoeningen. Daarnaast tonen onze resultaten aan dat LRP4 een veelbelovend target is voor de ontwikkeling van nieuwe behandelingen voor osteoporose. Daarenboven leverde dit project de basis voor het genereren van een *in vivo* muismodel voor sclerosteosis wat zal bijdrage tot de opheldering van de rol van LRP4 de botbiologie.

INTRODUCTION

CHAPTER 1

SCLEROSING BONE DYSPLASIAS: LEADS TOWARDS NOVEL OSTEOPOROSIS TREATMENTS

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ABSTRACT

Sclerosing bone dysplasias are a group of rare, monogenic disorders characterized by increased bone density resulting from the disturbance in the fragile equilibrium between bone formation and resorption. Over the last decade major contributions have been made towards better understanding of the pathogenesis of these conditions. These studies provided us with important insights into the bone biology and yielded the identification of numerous drug targets for the prevention and treatment of osteoporosis. Here, we review this heterogeneous group of disorders focusing on their utility in the development of novel osteoporosis therapies.

1 INTRODUCTION

The strength and shape of the skeletal bones are sustained by the fine balance between bone formation and resorption. Disruptions of this equilibrium may lead to pathologies characterized by abnormally increased or reduced bone mineral density (BMD). From these pathologies osteoporosis, defined by the BMD t-score equal or below -2,5, emerges as the heaviest socioeconomic burden on our population (1). Based on the combination of its high prevalence and the high heritability of relevant skeletal features, estimated at 25-85% percent, it is clear that genetic factors contribute greatly to this complex and multifactorial condition (2). To date, over 60 genomic loci have been identified as associated with BMD in large genome-wide association study (GWAS) efforts, highlighting several key regulatory pathways in bone biology, such as canonical Wnt signaling, RANK-RANKL-OPG system and pathways regulating endochondral ossification. Despite the large number of loci identified by GWAS, only a small fraction (<6%) of the genetic impact on BMD can be explained by these findings (3). Moreover, over half of the identified loci highlight genes without a know function in bone. Furthermore, most of the associated variants can remain in linkage disequilibrium with the truly causal variants creating the need for thorough functional verification of highlighted genes. These challanges, together with the scarcity of informative, monogenic disease models for low BMD, comprising only osteoporosis pseudoglioma syndrome and some forms of osteogenesis imperfecta shifted the scientific attention towards investigation of disorders residing on the other side of the spectrum to better our understanding of bone biology. **Sclerosing bone dysplasias** are a group of rare, monogenic disorders characterized by pathological increase in bone density resulting in a broad set of radiological and clinical abnormalities. These diseases are caused by a diverse spectrum of genetic factors. Over the years, studies unraveling these pathogenic mechanisms provided major contributions towards the better understanding of bone biology. Interestingly, some of the genes identified this way represented promising targets for the development of novel treatments for osteoporosis. In this review we summarize the major sclerosing bone dysplasias and discuss the lessons learned from the study of these rare disorders that contributed to current line of osteoporosis management.

2 BONE DISORDERS WITH INCREASED BMD

2.1 Sclerosing bone dysplasias caused by decreased bone resorption

One possible cause of elevated BMD is the impairment of bone resorption. As nicely demonstrated by the heterogeneous group of **Osteopetroses**, this can be due to impaired osteoclast differentiation or function. Two major modes of inheritance have been described for these conditions: autosomal recessive in case of severe forms, and autosomal dominant, usually leading to relatively mild forms. **Autosomal Recessive Osteopetrosis (ARO)** is often diagnosed shortly after birth and is characterized by a general increase of BMD, “Erlenmeyer flask” shape bones that display loss of trabecular structure and bone marrow failure due to the reduced bone marrow space. Neurological complications occur due to sclerotic changes at cranial foramina. The thorax is often small and sometimes hypertelorism, exophthalmos and micrognathia are observed. Increased propensity to fractures is a consequence of brittle bone structure. ARO is predominantly (~50% of cases)

caused by mutations in *TCIRG1* encoding for a subunit of vacuolar H^+-ATPase that transports protons into the resorption lacuna during the process of acidification of this compartment (4, 5).

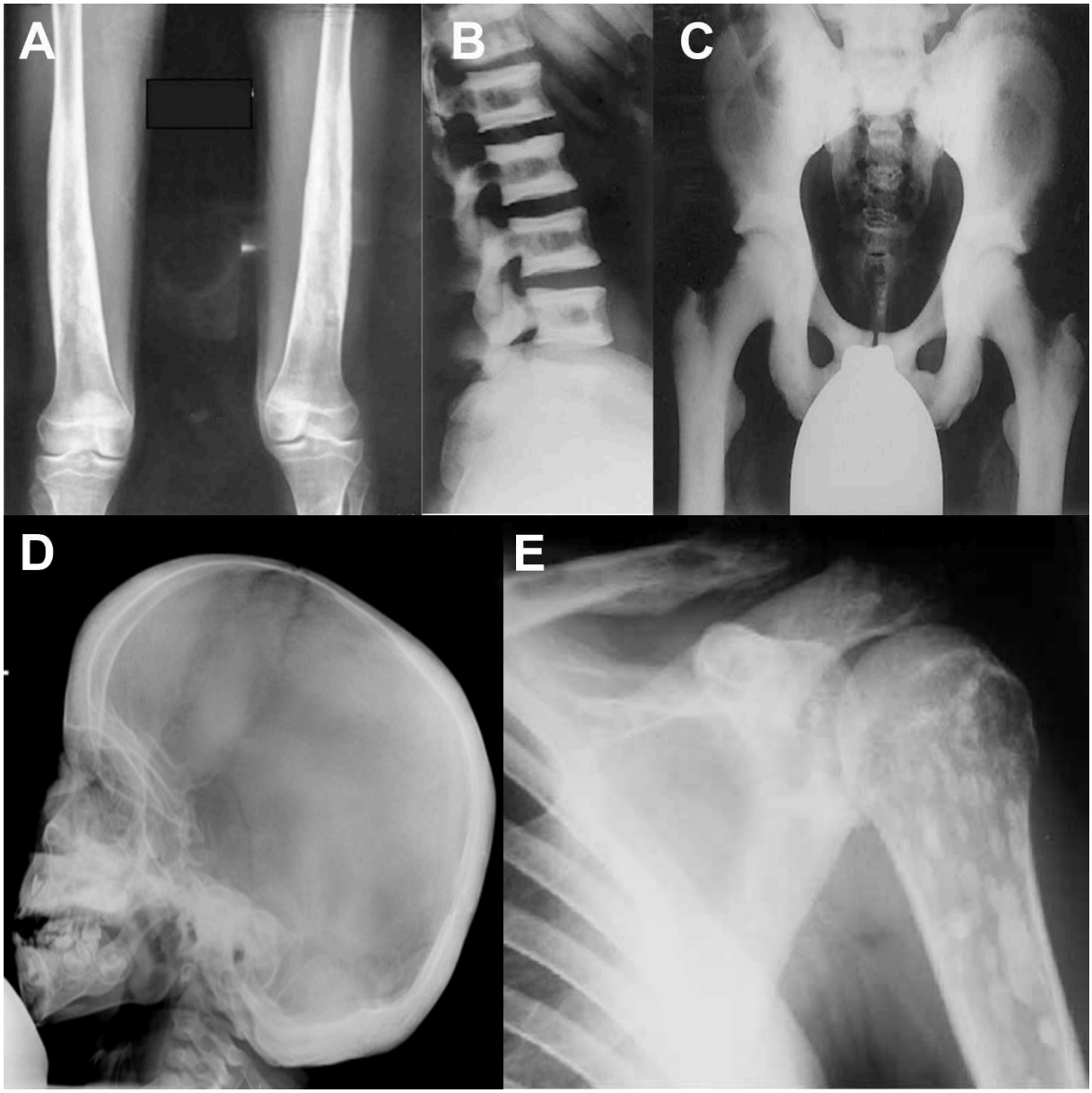

Figure 1

Characteristic features of selected osteochondrodysplasias. A-Camurati-Engelman disease. Cortical thickening of the diaphysis of the long bones with characteristic sparing of the metaphysis and diaphysis. B,C- ADOII. Sandwitch-like vertebrae due to sclerosis of the end plates (B). Pelvis of the patient with typical "bone within bone" appearance of the iliac wings (C). D-Craniometaphyseal dysplasia. Craniofacial sclerosis in a three-year-old patient. E-Osteopoikilosis. Characteristic lesions in the bone forming "spotted bone" appearance.

	Disease	Form	Genes	Mutation	Function	Radiological features
Decreased bone resorption	Osteopetrosis	X-linked	IKBKG	hypomorphic	OC differentiation	Optional bone thickening
		ARO	TNFSF11 TNFSF11A CAII TCIRG1 CLCN7 OSTM1	loss of function loss of function loss of function loss of function loss of function loss of function	OC differentiation OC differentiation Acidification by OC Acidification by OC Acidification by OC Acidification by OC	General ↑bone density, sclerosis at cranial base, "Erlenmeyer flask" deformity of bones, loss of trabecular structure, poor definition between cortex and medulla
		IARO	PLEKHM1 CLCN7 SNX10	loss of function hypomorphic loss of function	Acidification by OC Acidification by OC Acidification by OC	General sclerosis of the skeleton, widened metaphyses
		ADO	CLCN7	dominant negative	Acidification by OC	General sclerosis predominantly at vertebral endplates, iliac wings and the skull base
	Pycnodysostosis	AR	CTSK	loss of function	Collagenase activity	General sclerosis, short stature, dolichocephaly, open fontanel, clavicular dysplasia, obtuse angle of the mandible, short terminal phalanges
Increased bone formation	High bone mass phenotype	AD	LRP5	gain of function	Wnt signaling coreceptor	↑bone density, cortical hyperostosis at cranium, mandible and tubular bones
	Sclerosteosis	AR	SOST LRP4	loss of function loss of function	Wnt signaling inhibitor	Hyperostosis at calvaria, skull base, mandible and tubular bones; syndactyly, tall stature
	Van Buchem disease	AR	SOST	52kB deletion supressing SOST expression	Wnt signaling inhibitor	Hyperostosis at calvaria, skull base, mandible
	Craniodiaphyseal dysplasia	AD	SOST	loss of function	Wnt signaling inhibitor	Facial distortion, hyperostosis and sclerosis of cranial bones ("leontiasis ossea")
	Osteopathia striata (with cranial sclerosis)	X-linked	WTX	loss of function	Wnt signaling inhibitor	Metaphyseal striations, sclerosis at the skull base, absent fibulae
	Craniometaphyseal dysplasia	AD and AR	ANKH GJA1	loss of function (not yet known)	OB differentiation (not yet known)	Facial distortion, hyperostosis and sclerosis of cranial bones
	Camurati-Engelmann disease	AD	TGFβ1	gain of function	OB differentiation/proliferation	Cortical thickening, sclerosis of the diaphysis of the long bones by endosteal and periosteal proliferation, sclerosis of the basilar portions of the skull
	Osteopoikilosis/Buschke-Ollendorff syndrome	AD	LEMD3	loss of function	TGFβ and BMP signaling inhibitor	"Spotted bone"- small ovoid or lanceolate sclerotic lesions,
	Raine syndrome	AR	FAM20C	loss of function	OB differentiation	Severe facial distortion, generalized bone sclerosis
Increased bone turnover	Paget's disease of bone	AD	SQSTM1 VCP	gain of function	OC function	Focal abnormalities at one or multiple skeletal sites, bone deformities,
	Osteoectasia with hyperphosphatasia (Juvenile Paget's)	R	TNFRSF11B	loss of function	OC function	"Bowing" bones , short stature, kyphoscoliosis
	Familial expansile osteolysis	AD	TNFRSF11A	gain of function	OC function	Focal lesions, extreme bone deformities
	Expansile Skeletal Hyperphosphatasia	AD	TNFRSF11A	gain of function	OC function	Focal lesions, bone deformities

Table 1

Summary of main sclerosing bone dysplasias including mode of inheritance, causative genes, type of mutations identified in given genes, functions of causative genes and radiological hallmarks of the disorders; ARO (autosomal recessive osteopetrosis), IARO (intermediate autosomal recessive osteopetrosis), ADO (autosomal dominant osteopetrosis), AR (autosomal recessive), AD (autosomal dominant), OC (osteoclast), OB (osteoblast); Gene names were explained in the text.

Around 15% of ARO cases are caused by loss of function mutations in the *CLCN7* gene encoding the chloride channel crucial for the maintenance of the electric charge on both sides of the ruffled border (5). Deactivating mutations in another subunit of this protein complex, namely OSTM1, give rise to up to 6% of ARO (7). Another rare cause of ARO is a loss of function mutation in the gene encoding carbonic anhydrase II (*CAII*) that hinders the intracellular production of protons required for acidification of resorption lacuna (8).

In a subset of cases a reduction in the number of osteoclasts is observed causing a relatively slower disease progression and milder clinical manifestation. These, so called, **osteoclast poor forms of osteopetrosis** arise mainly from mutations in *TNFSF11* and *TNFSF11A* coding for receptor activator of NF-κB ligand and its receptor respectively (9, 10). Disruption of this pathway leads to impairment of osteoclast activation and differentiation. Hypomorphic mutations in this pathway (the inhibitor of nuclear factor kappa-B kinase subunit gamma – *IKBKG*) also lead to an X-linked form of osteopetrosis, namely **anhidrotic ectodermal dysplasia and immunodeficiency**. (11). A less severe clinical picture is also observed in the **intermediate autosomal recessive form (IARO)**. Short stature, delayed dentition and increased fracture risk are not accompanied by severe symptoms nor neurological complications. It has been shown that these forms of osteopetrosis are caused by hypomorphic mutations in CLCN7 and loss of function mutations in *PLEKHM1* (12). In addition, missense mutations in *SNX10,* coding for sorting nexin 10 protein which is crucial for endosomal trafficking within the osteoclast have been identified in patients suffering from IARO (13).

The **autosomal dominant form of osteopetrosis (ADO)** can also result from mutations in *CLCN7* but it is believed that in these patients functional complexes of a mature chloride channel are formed from both normal and mutant proteins resulting in only partial disruption of ion transport (14). Recently a number of patients diagnosed with **ADO (type 1)** were shown to carry mutations in LRP5 gene, a coreceptor of Wnt-signaling. Therefore, this condition is no longer considered part of the group of the osteopetroses as being caused by increased bone formation (15).

Mutations in the main cysteine protease of the osteoclast- Cathepsin K compromise collagenase activity of the cell and result in **Pycnodysostosis**- a rare, autosomal recessive disorder. Patients display short stature, open fontanel, clavicular dysplasia in addition to generally elevated bone density and risk of fracture. The osteocytes in patients suffering from this disease appear normal, however intracellular depositions of undegraded collagen are observed due to the lack of relevant enzymatic activity (16).

2.2 Sclerosing bone dysplasias caused by enhanced bone formation

Sclerosing bone dysplasias may also result from increased osteoblastic activity. Autosomal dominant **High bone mass phenotype (HBM)** is a mild example of such disorder with patients

presenting with elevated BMD, cortical thickening in cranial and tubular bones and resistance to fractures. It has been shown that the disorder is caused by activating mutations in the *LRP5* gene serving as a coreceptor for Wnt signaling (17, 18). The role of this pathway in bone biology was also elucidated by the discovery of loss of function mutations in the same gene causing Osteoporosis Pseudoglioma Syndrome characterized by early onset osteoporosis (19). A number of other *LRP5* mutations has been found to underlay other conditions characterized by abnormally elevated BMD such as endosteal hyperostosis, Worth syndrome or osteosclerosis. These results highlight Wnt signaling as one of the key players in osteoblastic differentiation, activation and survival. **Sclerosteosis** originates from loss of function mutations in the gene encoding sclerostin, a potent Wnt signaling inhibitor (20, 21). Sclerosteosis patients present with tall stature, syndactyly and generalized sclerosis of the skeleton, especially the skull bones and mandible. Some patients suffer from neurological complications such as cranial nerve palsies due to the sclerosis of the skull base or cephalgia caused by increased intracranial pressure. In 2011, we demonstrated that mutations in another Wnt-signaling coreceptor, namely LRP4, can also cause sclerosteosis (22). LRP4, has been shown to facilitate sclerostin inhibitory action and therefore partial loss of function mutations in this gene result in a similar phenotype as the mutations in *SOST*. A related, yet milder condition, is **Van Buchem Disease (VBD)**. Patients suffering from this rare, autosomal recessive disorder display sclerosis of the skeleton, most prominent in the skull bones, and show progressive enlargement of the mandible. The disease is caused by a 52kb deletion of a regulatory element localized 35kb downstream from the *SOST* gene, resulting in decreased production of sclerostin (23). Recently, mutations in *SOST* gene have also been described as causative for **craniodiaphyseal dysplasia**, a severe disorder marked by typical facial distortion termed “leontiasis ossea”. These mutations have been shown to largely impair sclerostin secretion (24).

Osteopathia striata is another sclerosing bone disorder, however with X-linked dominant mode of inheritance. This disease usually results in fetal or neonatal death in males, while females display longitudinal striations in the submetaphyseal regions of long tubular bones, pelvis and scapula. Clinical findings include cleft palate, hearing loss and macrocephaly. Causative mutations have been found in the *WTX* gene encoding a Wnt-signaling inhibitory protein capable of binding β-catenin (25). The phenotypic variability amongst affected females is most likely due to non-random X-inactivation.

Individuals with **Craniometaphyseal dysplasia** usually show a peculiar face with hypertelorism and a thick bony wedge over the bridge of the nose and glabella. Narrowing of the nasal passages may result in mouth breathing. Frequently, signs of cranial nerve impingement are seen with hearing loss, impaired vision or facial paresis. Mutations in the *ANKH* gene encoding a membrane transporter of pyrophosphate have been shown to cause the milder and more common autosomal dominant form of the disease (26, 27). Pyrophosphate is believed to inhibit mineralization of the bone matrix; therefore mutations in the gene might impair the transporter role of ANKH. Moreover, ANKH has also been shown to stimulate osteoblastic maturation and differentiation. Recently, mutations in the *GJA1* gene, encoding connexin 43, have been identified in patients suffering from the autosomal recessive form of the disease (28).

Camurati-Engelmann disease is a rare, autosomal dominant condition characterized by muscular weakness and leg pain in affected individuals. Moreover, cortical thickening of the long bones and hyperostosis of the skull base is observed. Camurati-Engelmann disease is caused by activating mutations in *TGFβ1*. Normally, TGFβ1 is stored in the bone in an inactive form due to its binding with latency-associated protein (LAP) (29, 30). Resorbing osteoclast releases the complex from the bone tissue initiating the migration of mesenchymal stem cells and their differentiation towards osteoblasts. With mutations disrupting the binding between TGFβ1 protein and LAP, this controlling process is disabled which may lead to pathologically up-regulated bone formation.

Mutations in *LEMD3*, a nuclear membrane protein that antagonizes both the TGFβ and BMP signaling pathways, have been identified as causative for **Osteopoikilosis** (31, 32). This autosomal dominant skeletal dysplasia is largely asymptomatic with radiological features including small, focal lesions at one or multiple skeletal sites. If the bone phenotype is accompanied by connective tissue nevi or juvenile elastoma, the condition is referred to as the-**Buschke-Ollendorff** syndrome.

Another disease caused by enhanced bone formation is **Raine syndrome**. This rare, severe syndrome usually results in death within the first weeks of life (mainly due to choanal atresia/stenosis). Surviving patients suffer from generalized increase in BMD, especially prominent at skull bones and severe facial distortion. Mutations in *FAM20C* gene encoding for a Golgi casein kinase protein have been identified in patients with this disorder (33). The protein has been shown to be crucial in the differentiation process of osteoblasts (34).

2.3 Sclerosing bone dysplasias with Increased bone turnover

As bone resorption remains tightly coupled with bone formation some disorders display elevated levels of both processes. Such is the case in **Paget's disease of bone** (PDB) where defective, numerous osteoclasts are accompanied by elevated osteoblastic activity. As a result of that, disorganized and weak bone tissue is produced. The typical age of disease onset situates within the 5^{th} or 6^{th} decade of life. Patients suffer from focal lesions affecting one or more skeletal sites, bone pain, increased incidence of fractures, bone deformities and elevated risk of developing osteosarcoma (35). So far, mutations in *SQSTM1* (sequestosome 1) and *VCP* (valosin containing protein) have been discovered in PDB patients suggesting the possible involvement of autophagy in the pathogenesis of the disease (36-38).

Another example of disease with disturbed bone turnover is **Juvenile Paget's disease** (Osteoectasia with hyperphosphatasia) marked by severe malformations of the skeleton with "bowing bones", short stature and kyphoscoliosis. Inactivating mutations in *TNFRS11B*, coding for osteoprotegerin (OPG) have been identified in this disease (39). Activating mutations in *TNFRSF11A* coding for RANK have been identified in a rare autosomal dominant disorder named **Familial expansile osteolysis** resulting in the same pathogenic mechanism (40). First hallmarks of the disease include hearing impairment and premature loss of dentition. Focal lesions, severe bone deformities, bone pain and frequent fractures appear early in life, usually between 15 and 45 years of age. In addition to that mutations in the gene encoding RANK have been found to be causative for **Expansile skeletal hyperphosphatasia** (41). The disease is

characterized by a progressive, generalized hyperostosis, early onset deafness and loss of dentition. Extreme bone pain usually occurs around adolescence affecting mainly the hands (42).

2.4 Sclerosing bone dysplasias with unknown genetic cause

Although many genes involved in the development of sclerosing bone disorders are already discovered as demonstrated above, there are also a number of patients diagnosed with sclerosing bone disorders with unknown genetic cause. This group includes both patients with a clear-cut diagnosis but without a mutation in the causative genes as well as patients with disorders for which the responsible gene is yet to be determined. As examples of the former, there are still several cases diagnosed with sclerosteosis, high bone mass phenotype, endosteal hyperostosis or different forms of osteopetrosis without mutations in the known genes (Table 1) (43-45). This can be due to locus heterogeneity or to misdiagnosis, as described recently for some cases of osteopetrosis by Pangrazio and colleagues (45). On the other hand there are still several sclerosing bone disorders for which no causative genes are known despite some attempts to identify mutations. Fortunately, novel technologies like next generation sequencing will help with the identification of the causative genes in the yet molecularly unsolved bone dysplasias.

Next generation sequencing technologies have already proven to be successful in the gene discovery of many skeletal dysplasias which is nicely reviewed by Lazarus et al (46).

Hyperostosis cranialis interna is a rare autosomal dominant disorder which is characterized by intracranial hyperostosis and osteosclerosis of the skull. Linkage analysis in one Dutch family demonstrated recently that the causative gene is located in a region on chromosome 8p21 encompassing 64 genes, however the causative mutation is still to be identified (47). **X-linked calvarial hyperostosis** is a very rare sclerosing bone disorders only affecting the skull. Only one family is described so far by Pagon and colleagues in 1986 but the causative gene is yet to be identified (48, 49). Another sclerosing bone dysplasia with unknown cause is **melorheostosis**. It is characterized by asymmetric hyperostotic lesions in the cortex of tubular bones. The lesions usually affect one limb and besides the bone also other adjacent tissues can be affected (31). Melorheostosis is, albeit rarely seen in families with osteopoikilosis and consequently, it was suggested that germline or somatic mutations in LEMD3 can be the cause for this disorder, however, several studies were unable to confirm this (31, 32, 50). Finally, identification of the genetic cause of above described disorders and several other unidentified disorders such as for example **Pyle disease** and **osteomesopyknosis** will increase the insights in bone biology greatly which is interesting for the development of novel agents for treatment of common bone disorders such as osteoporosis (51, 52).

3 TARGETS FOR OSTEOPOROSIS TREATMENT

As mentioned before, osteoporosis is a common disease with a high socioeconomic impact. Nowadays, bisphosphonates are widely used for osteoporosis treatment. Bisphosphonates can

bind to bone and after internalization by the osteoclasts, they are able to prevent further bone resorption and bone loss. However, prevention of bone loss is shown to be insufficient for the prevention of osteoporosis related fractures. Genetic studies identifying the cause of monogenic sclerosing bone dysplasias have not only provided major insights into the bone biology, but also have highlighted novel pathways and sites of potential pharmacological intervention. Over the years a list of such findings has been translated into therapeutic strategies for management of osteoporosis (Table 2).

3.1 Inhibition of bone resorption

Identification of the genetic cause of osteopetrosis increased our insights into osteoclastogenesis and osteoclast function. In this way the RANK-RANKL-OPG pathway has been described as an important regulator of osteoclast formation and function. Binding between RANK and **RANKL** leads to osteoclast activation and is regulated by osteoprotegerin (OPG), an inhibitor of the pathway secreted by osteoblasts (Figure 2). Denosumab is a monoclonal humanized RANKL antibody mimicking the action of OPG and in this way preventing bone loss. Initially denosumab was approved by the FDA in 2010 for the treatment of postmenopausal osteoporosis. More recently, it is also approved for treatment in men with high risk of fracture (53). Finally, a combined treatment with denosumab and teriparatide was recently evaluated in a two-year randomized trial in osteoporotic women. This combined therapy seems promising and showed a significant increase in spine, femoral neck and hip BMD in comparison to the use of a single therapeutic agent (54).

CLCN7 is another target for osteoporosis treatment that is identified through the study of causative genes for osteopetrosis. CLCN7 is a chloride channel present in the osteoclasts and important for acidification of the resorption lacunae. In 2004, NS3736 was identified as possible drug for osteoporosis treatment by inhibiting the osteoclastic chloride channel encoded by *CLCN7*. In ovariectomized rats, it was shown that NS3736 inhibits bone resorption without affecting bone formation (55). However, further studies are needed to evaluate efficacy and safety of this small molecule. In addition to mutations in CLCN7, mutations in ***TCIRG1*** are also shown to be causative for autosomal recessive osteopetrosis. Furthermore, *TCIRG1* encodes a subunit of the osteoclast specific vacuolar H^+-ATPase which is important for acidification of the resorption lacunae. Several inhibitors of the V-ATPase activity are described for example Bafilomycin A1, Concanamycin A, SB242784, FR167356 and FR202126, however, the available experimental data regarding treatment of osteoporosis for all components is limited and more research is needed regarding specificity, efficacy and safety (56).

Pycnodysostosis is another sclerosing bone disorder caused by defects in osteoclast function. Nonsense mutations in ***CTSK***, a lysosomal protease released by the osteoclast, are shown to be causative for the increase in bone mass seen in these patients (16). The role of cathepsin K was also confirmed by the osteopetrotic phenotype of the *ctsk* knockout mouse (57). Based on these data, cathepsin K was considered as an interesting target for osteoporosis treatment. As a consequence, several inhibitors were developed but clinical trials for most agents are stopped as a result of adverse reactions or lack of selectivity. The most promising inhibitors which are

still under study are Odanacatib, ONO-5334 and MIV-711 (58). Clinical studies investigating the effect of Odanacatib on BMD are most advanced and have reached phase III. Results of the phase II clinical trials show that Odanacatib reduces bone resorption by blocking osteoclast function without affecting differentiation or survival. Finally, Odanacatib does not affect bone formation indicating that bone resorption and formation are uncoupled (59). Although Odanacatib treatment looks promising, more studies are needed to determine its effect on fracture risk and safety (60).

3.2 Increasing bone formation

The importance of Wnt signaling in the regulation of bone formation was highlighted by genetic studies unraveling the genetic cause of the high bone mass phenotype, sclerosteosis and Van Buchem disease. Relevance of the pathway in the development of novel osteoporosis therapies was extensively discussed elsewhere, here we focus on therapies emerging from genes highlighted by the studies in sclerosing bone dysplasias (61-63). Loss of function of **sclerostin** causes both sclerosteosis and Van Buchem disease. Furthermore, sclerostin is almost exclusively expressed in the osteocytes and *Sost* knockout mice have an increased bone mass. These findings point to sclerostin as a promising drug target for osteoporosis. Several pharmaceutical companies are developing sclerostin antibodies (Romosozumab, Blosozumab and BPS804) as treatment for osteoporosis. Most advanced are the studies of Romosozumab (Amgen), a humanized monoclonal antibody targeting sclerostin which entered phase III of clinical trials. In a recent phase II study, it was shown that monthly subcutaneous injection of sclerostin results in an increased BMD at several sites in post-menopausal women with low bone mass. Based on bone turnover markers the study demonstrated that Romosozumab effects bone formation rapidly and clear, however, the effect is transient. This is in contrast with the effect on bone resorption which is moderate but continuous during the period of treatment (64).

Besides a function of sclerostin antibodies in the treatment of postmenopausal osteoporosis, the efficacy of these antibodies is also tested in several animal models with monogenic osteoporosis. Both in a model for osteoporosis pseudoglioma (*Lrp5*$^{-/-}$ mouse) and in a model for osteogenesis imperfecta (*Brtl*$^{/+}$ mouse) inhibition of sclerostin can improve bone mass and decrease fractures (65, 66). The effect of complete deletion of sclerostin in the OPPG mouse was also studied in an *Lrp5*/*Sost* double knockout mouse model. These double knockout mice have larger and stronger bones than *Lrp5*$^{-/-}$ mice, indicating that sclerostin acts also through LRP5 independent pathways to increase bone mass.

In addition to sclerostin, there are several other modulators of the canonical Wnt signaling pathway. Recently, it was shown that both in patients and mice lacking sclerostin, expression of dickkopf1 **(DKK1)**, another inhibitor of the pathway, is upregulated (67, 68). Complete deletion of *dkk1* in mice is lethal, but a heterozygous *dkk1*$^{+/-}$ mouse model has an increased bone mass (69). These data indicated that DKK1 is an interesting target for the treatment of bone disease. Fully human monoclonal DKK1-neutralizing antibodies are currently under study in several animal models for OVX-induced osteoporosis, multiple myeloma and erosive rheumatoid arthritis with promising results. However, more studies are needed. Especially for

the management of multiple myeloma, DKK1-antibody treatment (BHQ880, Novartis) looks promising and clinical trials are ongoing (clinicaltrials.gov).

Next to treatments developed to target genes highlighted by sclerosing bone dysplasias other known modulators of Wnt signaling were evaluated for their potential therapeutic relevance.

Agent	Current stage	Targeted mechanism	Mechanism of action
RANKL-antibodies (denosumab)	Marketed in 2010	OPG/RANK/RANKL pathway	Binds to RANKL and mimics the effect of OPG preventing RANK activation and subsequent stimulation of osteoclast differentiation, activation and survival.
CLCN7 inhibitors (NS3736)	Preclinical	Osteoclastic bone resorption	Inhibits the acidification of resorption lacunae by blocking chloride ions transport.
Vacuolar ATPase inhibitors (SB242784, FR167356, FR202126)	Preclinical	Osteoclastic bone resorption	Inhibition of resorption lacunae acidification by disruption of main osteoclastic ATP-dependent proton pump.
Cathepsin K inhibitors (Odanacatib, ONO-5334, MIV-711)	Phase III clinical trials	Osteoclastic bone resorption	Inhibition of main cysteine protease of the osteoclast used for bone matrix proteins degradation.
SOST-antibodies (Romosozumab, Blosozumab, BPS804)	Phase III clinical trials	Wnt signaling	Neutralization of Wnt signaling inhibitor that is selectively secreted by osteocytes. Upregulation of the pathway enhances bone formation.
DKK1-antibodies (BHQ880)	Preclinical	Wnt signaling	Neutralization of secreted Wnt signaling inhibitor. Upregulation of the pathway enhances bone formation.

Table 2
Summary of osteoporosis drugs targeting mechanisms highlighted by the research in sclerosing bone dysplasias.

An example of such effort is a series of *ex vivo* studies by Moore et al. demonstrating that inhibition of secreted frizzled-related protein 1 **(sFRP1)**, another inhibitor of the pathway, by small molecules might be a base for future therapy for osteoporosis (70, 71).

In conclusion, it is clear that the identification of genes responsible for monogenic sclerosing bone disorders not only increase the knowledge on pathways involved in the regulation of bone remodeling but also open new therapeutic avenues for the treatment of patients with osteoporosis.

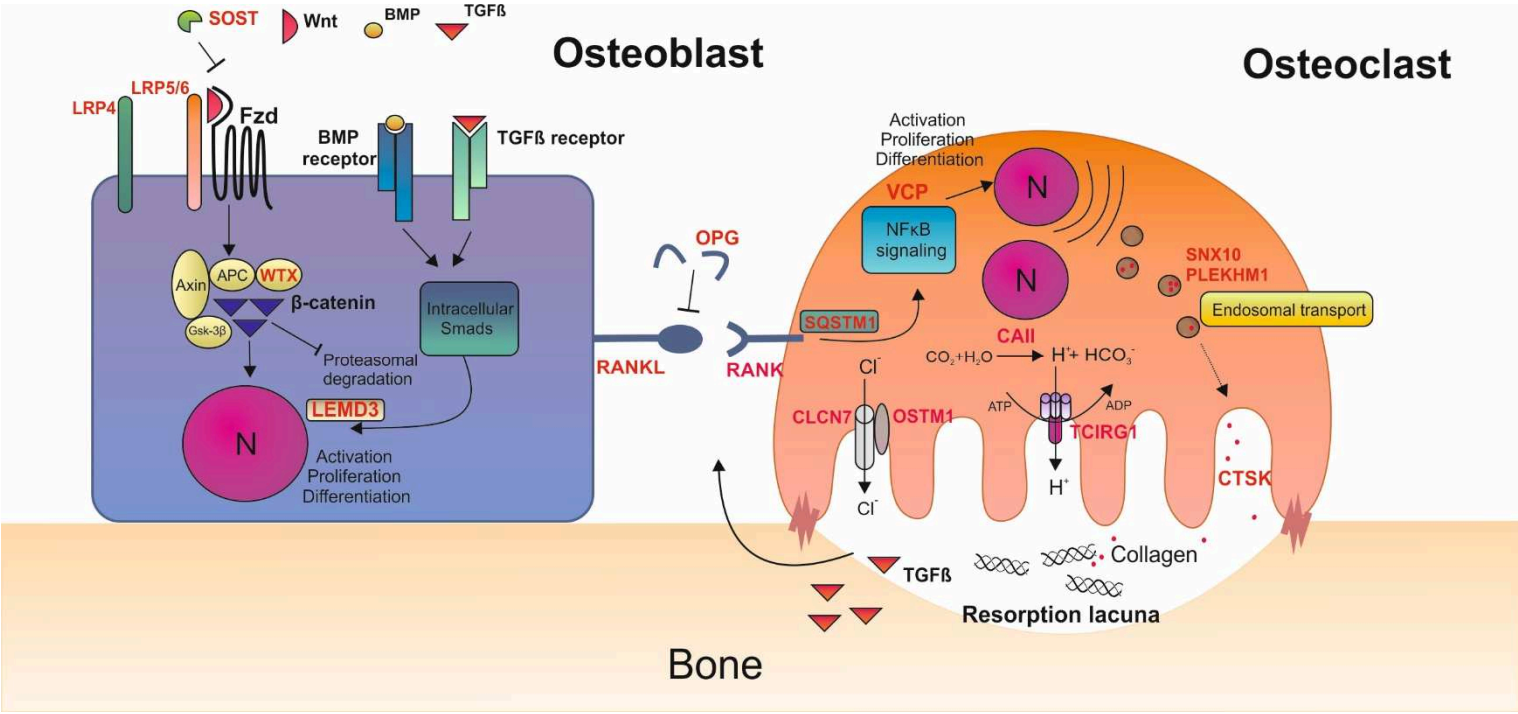

Figure 2

Overview of pathways and mechanisms involved in the pathogenesis of sclerosing bone dysplasias. Gene names were discussed in the text.

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CHAPTER 2

THE ROLE OF EXTRACELLULAR MODULATORS OF CANONICAL WNT SIGNALING IN BONE METABOLISM AND DISEASES

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ABSTRACT

Objectives: The Wnt signalling pathway is a key pathway in various processes, including bone metabolism. In this review, current knowledge of the canonical Wnt signalling and its extracellular modulators in bone metabolism is summarized and discussed.

Methods: The PubMed database was searched using following key words; canonical Wnt signalling, β-catenin, Wnt signalling modulators, bone metabolism, BMD, osteoblast, osteoporosis, Wnt, LRPs, Frizzleds, sFRPs, sclerostin or *SOST*, dickkopfs, Wif1, R-spondins, glypicans, SOST-dc1 and kremen all separately as well as in different combinations.

Results: Canonical Wnt signalling is, since the identification of *LRP5* and *SOST* as disease causing genes for several monogenic sclerosing bone disorders, considered to be the major pathway regulating bone formation. Therefore, a large number of studies were performed to elucidate the role of numerous proteins in canonical Wnt signalling and bone metabolism. These studies led to novel insights in the regulation of canonical Wnt signalling. Consequently, several proteins involved in this pathway are interesting targets for the development of novel therapies for osteoporosis and other bone diseases. However, the pleiotropic function of the pathway still remains a big concern when developing novel drugs.

Conclusion: It is clear that canonical Wnt signalling has an important role in the regulation of bone metabolism. The increasing number of studies into the exact function of all proteins in the canonical Wnt pathway in general and in bone metabolism already led to increased knowledge and novel insights in the regulation of the canonical Wnt pathway.

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1. INTRODUCTION

Wnts are a family of cysteine-rich, secreted glycoproteins functioning in a plethora of cellular actions, including embryonic development, postnatal development, induction of cell polarity, maintenance of tissues homeostasis and cell growth control can be listed (1). In mammals, 19 of these growth factors have been identified to date (2). Wnts signal transduction is mediated through interaction with one of ten Frizzled (Fz) receptors and if necessary through interaction with additional co-receptors like low density lipoprotein receptor related protein 5 or 6 (LRP5/6) (Figure 1). Among numerous possible combinations at least three signalling pathways can be distinguished but cross-talk between them can occur at some level (3).

The best understood pathway is the Wnt/β-catenin signalling (canonical) pathway. Binding of the "canonical" Wnt ligand (e.g., Wnt 1, 3a, and 8) to the Fz receptor-LRP5/6 receptor complex initiates Disheveled protein (Dvl)-dependent inhibition of glycogen synthase kinase 3β (GSK3β) complex activity, thus protecting β-catenin from phosphorylation and subsequent degradation. Stabilized β-catenin accumulates in the cytosol and translocates into the nucleus, where it associates with lymphoid-enhancer binding factor (Lef)/ T-cell specific transcription factors (Tcfs). Such interaction results in transcription of target genes (Figure 1) (4-6).

"Non-canonical" Wnts (e.g., Wnt 5a, 11) lack the ability to affect β-catenin levels and trigger two alternative signalling pathways. First of them, the Ca^{2+} dependent pathway leads to release of intracellular calcium and, in consequence, activation of calcium sensible enzymes. Among them Ca^{2+}-calmodulin dependent kinase II (CamKII), protein kinase C (PKC) and calcineurin (CaCN) have been identified (Figure 1). A cascade of events ultimately culminates in modulation of cell migration, dorso-ventral patterning of the embryo and heart development (7). Secondly, the planar cell polarity (PCP) pathway involves the action of Dvl protein and activation of Rho and Rac GTPases which results in the activation of c-jun NH2-terminal kinase (JNK) (Figure 1) (8, 9). This contributes to the control of cell shape, cell fate determination and embryonic morphogenesis (10).

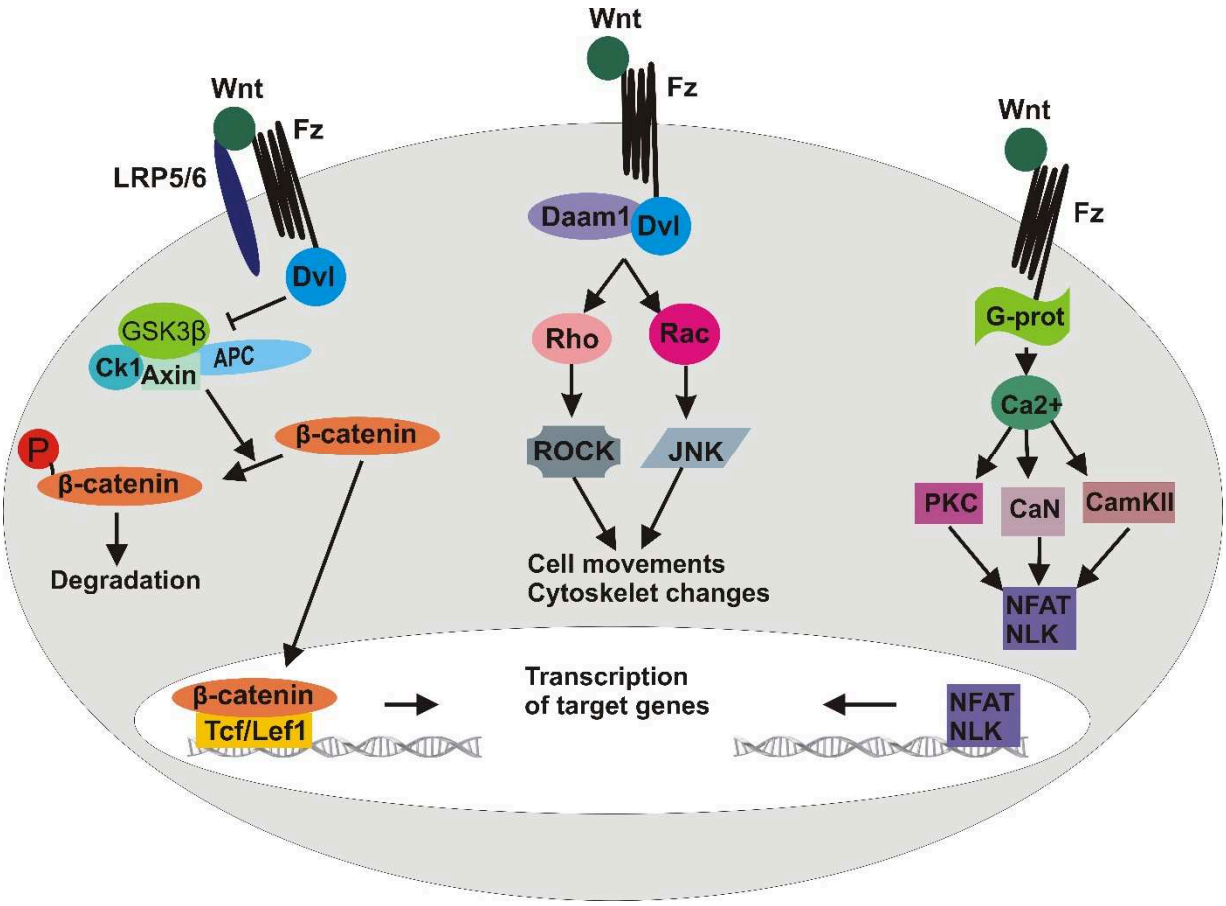

Figure 1: Overview of the three different Wnt signalling pathways. Activation of the canonical Wnt signalling pathway (left) results in inhibition of the protein complex formed by GSK3B, APC, CK1 and axin and consequently this results in increased B-catenin levels in the cytoplasm. Activation of the non-canonical PCP (middle) and calcium dependent (right) Wnt pathway by Wnt results activation of Rho/Rac GTPases and PKC/CaN/CamKII, respectively.

1.1. Proteins involved in Canonical Wnt signalling in bone metabolism

Since establishing the involvement of Wnt/β-catenin signalling in bone metabolism, major research efforts have been undertaken to elucidate the molecular mechanisms behind this pathway and its regulation. Here we only review extracellular and trans-membrane modulators of canonical Wnt signalling and bone metabolism.

1.2. Wnt ligands

Wnt ligands are secreted glycoproteins consisting of 350-400 amino acids of which 23-24 conserved cysteine residues. These cysteine residues are likely to participate in intramolecular disulfide bonds (3, 11). In humans the Wnt family consists of 19 members (12) which can activate the three Wnt signalling pathways by binding to Frizzled (Fz) and if necessary additional co-receptors (Firgure 1). The subdivision of Wnts in "canonical" or "non-canonical" Wnts which is previously used, originates from the early studies in *Xenopus* embryo's and is somewhat out of date since it is shown that a "non-canonical" Wnt can be turned into a

"canonical" Wnt when provided with the appropriate receptor (13, 14). In this regard, it was more recently suggested that the activation of downstream pathways actually relies on the receptor, and not on the Wnt itself (reviewed by van Amerongen and colleagues) (14).

As a consequence, the role of the different Wnt ligands in bone homeostasis and bone disease is not yet fully understood although several studies already identified important functions in bone metabolism via the canonical Wnt pathway for a large number of Wnts. Here, we summarize the role of the "canonical" Wnts Wnt3a, Wnt10b, Wnt14 and Wnt16 in bone metabolism. Furthermore, we discuss the role of the previously reported non-canonical Wnts, Wnt5a and Wnt11 in the regulation of canonical Wnt signalling and bone metabolism.

1.2.1. Wnt3a

Wnt3a is together with Wnt1 the most frequently used Wnt ligand for *in vitro* stimulation of canonical Wnt signalling in order to induce cell proliferation and survival in osteoblasts *in vitro* (15, 16). Recent studies showed that canonical Wnt signalling activation by Wnt3a results in inhibition of the ErbB3 receptor causing the stimulation of osteoblast differentiation of murine mesenchymal stem cells (17). In addition to these *in vitro* experiments, studies in humans and mice confirmed that *Wnt3a* is involved in regulating bone metabolism. The heterozygous deletion of *Wnt3a* in male mice results in a decreased bone mass (Table 1) (18).

In humans, a candidate gene association study demonstrated that two common SNPs in *Wnt3a* are associated with bone mineral density (BMD) at the hip in post-menopausal Caucasian women (19). In addition, a rare heterozygous variant (p.K51R/-) in Wnt3a was identified in patient with primary osteoporosis and his affected family members, but not in healthy family members (Table 1). Furthermore, functional studies showed that the Wnt signalling activity of the mutant Wnt3a was reduced (20).

1.2.2. Wnt10B

Wnt10b is one of the most extensively studied Wnt ligands, as well in bone disorders as in other diseases. The role of Wnt10b in several diseases is nicely discussed elsewhere by Wend *and colleagues* (21). Wnt10b acts through the canonical Wnt signalling and its function in bone metabolism is studied using different mouse models. Transgenic overexpression of *Wnt10b* in mature osteoblast, driven by an osteocalcin promoter (Oc-Wnt10b) and in bone marrow-derived mesenchymal precursor cells under control of a fatty acid-binding protein 4 (FABP4) promoter (FABP4-Wnt10b) both results in increased bone mass and bone strength (Table 1) (22, 23). The increased bone mass in Oc-Wnt10b mice, is caused by increased bone formation due to elevated osteoblast numbers (22). FABP4-Wnt10b mice are also shown to be resistant to age-related and hormonal related bone loss. Furthermore these mice have reduced adiposity and are resistant to obesity (22, 23). These results indicate that Wnt10b has an important function in regulating the differentiation of mesenchymal precursor cells into either osteoblast or adipocytes. In addition to the transgenic mouse models, *Wnt10b*$^{+/-}$ and *Wnt10b*$^{-/-}$mice were studied and showed a reduced bone mass due to decreased function and number of the osteoblast (Table 1) (24).

In humans, homozygous missense mutations in *Wnt10b* were demonstrated to cause split-hand/foot malformation (Table 1) (25-27). In addition, it was shown that the expression of

Wnt10b correlates with the survival rate in patients with osteosarcoma, a primary malignant tumor of the bone. This suggests that Wnt10b-induced Wnt signalling has a role in the progression of osteosarcoma (28). Finally, results of mouse studies suggested that Wnt10b is a susceptibility gene for osteoporosis. To confirm the role of Wnt10b in osteoporosis in humans, several candidate gene association studies were performed with *Wnt10b* polymorphisms and bone mass which led to contradicting results (Table 1) (19, 29, 30). As a consequence the influence of *Wnt10b* variation on osteoporosis susceptibility needs to be elucidated further.

1.2.3. Wnt14

Wnt14, also known as *Wnt9a*, clusters on chromosomal region 1q42 together with *Wnt3a* and is shown to activate canonical Wnt signalling (Table 1). *Wnt14* expression was demonstrated in tissue surrounding mesenchymal condensations and in differentiating osteoblasts (31-33). Several studies indicated that Wnt14 is involved in joint formation and maintenance and in skeletogenesis. Furthermore, in *zebrafish* a critical role for Wnt14 in craniofacial morphogenesis was demonstrated (34). In mice, endochondral bone formation was blocked by high transgene expression levels of *Wnt14*. However, lower transgene expression levels of *Wnt14* resulted in increased chondrocyte maturation and endochondral bone formation (Table 1) (35).

In addition to these results, *Wnt14* was suggested as a candidate gene for regulating BMD by a study combining linkage, gene expression and association data in mice. *In vitro* studies showed that *Wnt14* expression was downregulated during differentiation of mesenchymal stem cells to adipocytes (36). All data together strongly suggest that Wnt14 contributes to the regulation of bone formation.

1.2.4. Wnt16

Wnt16 expression was found in the joints, skin, during limb and craniofacial development and in the perichondrium and periosteum of the developing skeleton (32, 33). Furthermore, its expression was up-regulated in areas with moderate to severe osteoarthritis damage (37). Recently polymorphisms in or close to *Wnt16* were shown to be associated with BMD, peak BMD, fracture risk and cortical thickness in several large genome wide association studies (38, 39).

Previous to these studies, it was already demonstrated that the chromosomal region containing Wnt16, 7q31 influences hip and femoral neck BMD in Amish men (Table 1) (40).Furthermore, the role of Wnt16 in the regulation of BMD, fracture risk and cortical bone thickness was confirmed by studies in *wnt16* knockout mice (38, 39). These mice showed reduced total body BMD, thinner cortical bones and reduced bone strength (Table 1).

1.2.5. Wnt5a

Wnt5a was first classified in the group of non-canonical Wnts. However, it was more recently shown that Wnt5a can influence all Wnt pathways depending on the available Fz receptors (41). Furthermore recent studies demonstrated that Wnt5a can both activate and inhibit the canonical Wnt signalling pathway. *In vitro* studies showed that Wnt5a in the presence of Fz4 and LRP5,

can activate canonical Wnt signalling in multiple cell lines (41). Furthermore, activation of the canonical Wnt signalling was also demonstrated *in vivo* in the developing skull of *wnt5a* overexpressing mice (42). In contrast to these findings, an inhibitory role for Wnt5a on Wnt/β-catenin signalling was demonstrated both *in vitro* and *in vivo* (42). However, the mechanism whereby Wnt5a antagonizes canonical Wnt signalling is still unclear but seems to be mediated through the tyrosine kinase-like orphan receptor 2 *(*Ror2) (43-45). One study reported that overexpression of *Wnt5a* resulted in increased degradation of β-catenin while other studies showed that Wnt5a inhibition acts downstream of β-catenin at the level of TCF-transcription (41, 46).

In addition to these studies, *in vivo* studies in mice and human suggest that Wnt5a is involved in the regulation of bone metabolism. The results of these studies suggest that Wnt5a exerts its effect on bone metabolism mainly through the non-canonical Wnt signalling. In mice, heterozygous deletion of *Wnt5a* results in decreased BMD and increased adipogenesis (Table 1) (18). These findings were also confirmed by *in vitro* differentiation experiments with human mesenchymal stem cells which showed that in the absence of Wnt5a human mesenchymal stem cells will differentiate into pre-adipocytes (47). In humans, loss-of function mutations in *Wnt5a* result in dominant Robinow Syndrome (Table 1), a disease characterized by short stature, limb shortening, hypertelorism, mandibular hypoplasia, irregular dental alignment and hypoplastic external genitalia (48). In addition to the dominant form, a autosomal recessive form of Robinow syndrome exists which is caused by mutations in *ROR2*, a co-receptor for Wnt5a in the non-canonical Wnt signalling (49). In the future, supplementary studies unrevealing specific-receptor ligand interactions will contribute to the understanding of the complexities of Wnt signalling and the role of Wnt5a in Wnt signalling. Furthermore, it needs to be determined whether *in vivo* Wnt5a is able to activate Wnt/β-catenin signalling in physiologic conditions.

1.2.6. Wnt11

After its discovery in 1994, Wnt11 was classified in the group of non-canonical Wnts (50). However more recently it was shown that this classification is not always true and that Wnt11 has the potential to activate all pathways depending on the context (51). The mechanism whereby Wnt11 can activate canonical Wnt signalling is not clear but it seems to take place at several levels. Furthermore, it is demonstrated that Wnt11 like Wnt5a, can also be a potent inhibitor of Wnt/β-catenin signalling in different cell types which makes it even more complex. Consequently, it is suggested that Wnt11 alters the expression of canonical Wnts or Wnt antagonists which can affect canonical Wnt signalling (52).

Wnt11 is expressed in human mesenchymal stem cells and the expression was increased during osteogenic differentiation which indicates that Wnt11 has a potential role in osteoblast differentiation (16). This was confirmed *in vitro* in MC3T3-E1 cells were osteoblastogenesis was stimulated upon activation of canonical Wnt signalling by Wnt11 (53). Subsequent investigations showed that Wnt11 signals through β-catenin to activate R-spondin2 expression which is required for osteoblast maturation mediated by Wnt11 (53).

The viability of mice completely lacking *wnt11* after post-natal day 2 is only 20%. Most *wnt11*$^{-/-}$ mice die in a late embryonic or early post-natal phase because of cardiac dysfunction and have

no skeletal developmental abnormalities (52, 54). Wnt11$^{-/-}$ mice surviving post-natal day 2 have increased trabecular but not cortical bone mass compared to wild type at 4 and 8 months of age (Table 1). Analysis of β-catenin expression in mesenchymal stem cells and dermal fibroblasts of the wnt11$^{-/-}$ mice showed that β-catenin levels were increased which suggests that Wnt11 is an inhibitor of Wnt/β-catenin signalling. Additional evidence for an inhibitory function of Wnt11 on canonical Wnt signalling was delivered by expression studies during adipocytogenesis of mesenchymal stem cells. During the differentiation to adipocytes, *Wnt11* expression was increased and the expression of "canonical" Wnts was decreased. Furthermore, activation of Wnt/β-catenin signalling by inhibition of GSK3β inhibited adipocytogenesis and decreased expression of *Wnt11* (55). These findings were in contrast with the results of *in vitro* studies in MC3T3-E1 cells demonstrating that wnt11 activates osteoblastogenesis through canonical Wnt signalling (52, 56).

As a consequence, further studies are needed to elucidate the effect of Wnt11 on canonical Wnt signalling. It is possible that the effect is dependent on the available receptors as it is shown for Wnt5a (41).

Table 1: Chromosomal location (Chrom), mouse models and associated bone related diseases for Wnt3a, Wnt10b, Wnt14, Wnt16, Wnt5a and Wnt11. Tg: transgene overexpression. ↑: increased, ↓: decreased, (p)BMD: (peak) bone mineral density, Oc: osteocalcin, FABP4: fatty acid-binding protein 4

Wnt	**Chrom**	**Mouse Model**		**Associated human bone disease**		**Ref**
		Model	**Phenotype**	**Phenotype**	**Association / mutation**	
Wnt3a	1q42	*wnt3a*$^{+/-}$	↓ bone mass in ♂	Osteoporosis	SNPs associated with hip BMD Missense mutation p.K51R/-	[18-20]
Wnt10b	12q13	*wnt10b*$^{+/-}$, *wnt10b* $^{-/-}$	↓ BMD, ↑ adipogenesis	Osteoporosis	SNPs associated with BMD	[22-30]
		Tg: Oc-*wnt10b*	↑ BMD and bone strength	Split-hand/foot malformation	Homozygous missense mutations	
		Tg: FABP4-*wnt10b*	↑ BMD and bone strength, ↓adipogenesis Resistant for obesity and age related bone loss	Osteosarcoma	Expression correlates with survival rate	
Wnt14 (Wnt9a)	1q42	Tg: Col2a1-*wnt14*	High Tg expression: inhibition of the enchondral bone formation Low Tg expression: ↑chondrocyte maturation and enchondral bone formation			[35]
Wnt16	7q31	*wnt16*$^{-/-}$	↓BMD and bone strength, thinner cortical bones	Osteoporosis	SNPs associated with BMD, pBMD, fracture risk and cortical thickness	[38-40]
Wnt5a	3p21-p14	*wnt5a*$^{+/-}$	↓ BMD, ↑ adipogenesis	Robinow Syndrome	Loss-of-function mutations	[18, 48]
Wnt11	11q13.5	*wnt11*$^{-/-}$	Post-natal day 2 survival rate: 20% ↑ trabecular bone mass (4 and 8 months)			

Table 2: Chromosomal location (Chrom), mouse models and associated bone related diseases for Frizzled (Fz) 1, 2 and 6. Tg: transgene overexpression. ↑: increased, ↓: decreased

Fz	Chrom	Mouse model		Associated human bone disease		Ref
		Model	**Phenotype**	**Phenotype**	**Association / mutation**	
Fz1	7q21			Osteoporosis	Promoter SNP associated with fracture risk and femoral neck geometry only in men	[63, 64]
Fz2	17q21.1	Tg rat 3.6 Col1 L*RP5*^{G171V}	↑ *fz2* expression as response to mechanical loading			[65]
Fz9	7q11.23	*fz9*$^{-/-}$	↓ bone formation, osteopenia			[59]

1.3. Frizzled receptors

Frizzled proteins (Fz) are seven-pass membrane receptors which can activate both canonical and non-canonical Wnt signalling (Figure 1) (57). In humans the Fz family contains 10 members and it is suggested that not all Fz proteins have the same function in Wnt signalling indicating the existence of inhibitory and stimulatory Fzs. Wnt ligands can interact with Fz through a cysteine-rich domain at the extracellular surface. Little is known about the Wnt specificity and affinity of Fzs. However, it is expected that there is some redundancy since there are nearly twice as many Wnts as Fzs (58). Furthermore, information about the roles of specific Fzs in bone metabolism is currently lacking.

In literature, there is some evidence that Fz1, 2 and 6 are involved in the regulation of bone formation through canonical Wnt signalling. In addition, it is reported that deletion of *Fz9* in mice results in reduced bone mass. However, in these mice β-catenin levels were not altered which indicates that the influence of Fz9 on bone formation does not act through canonical Wnt signalling in these mice (Table 2) (59, 60).

1.3.1. Frizzled 1 (Fz1)

*Fz*1 expression is found in chicken during craniofacial development, in murine mesenchymal stem cells and in human osteosarcoma cells (58, 61). *In vitro*, murine *fz1* overexpression reduces canonical Wnt signalling in COS-7 cells as well as in a number of pluripotent mesenchymal cell lines (62). In these last cells, bone morphogenetic protein 2 (BMP-2) can upregulate the expression of *fz1* which results in an inhibition of the BMP-2 induced osteoblast differentiation. This antagonistic effect of murine fz1 on Wnt/β-catenin signalling is not demonstrated for human Fz1 (62).

An indication that human Fz1 is involved in fracture risk susceptibility is delivered by an association study in a large sample of Afro-Caribbean men (63). This study shows that common genetic variation in the *Fz1* promoter region is associated with femoral neck geometry parameters but not with BMD. In addition, a second study in post-menopausal Korean women did not find an association between one coding SNP in *Fz1* and BMD (64).

No data are available for the effect of common variation in *Fz1* on femoral neck geometry parameters in other populations (Table 2). As a consequence, more studies are needed to evaluate the role of Fz1 in Wnt signalling on bone metabolism in humans.

1.3.2. Frizzled 2 (Fz2)

Frizzled 2 (Fz2) expression was, similar to *Fz1,* shown during craniofacial development in chicken, in murine osteoblasts and in human osteosarcoma cells (58). A function for Fz2 in bone formation was demonstrated *in vivo* and *in vitro* by experiments studying the response to mechanical loading (65). Mechanical loading is shown to activate bone formation through the activation of canonical Wnt signalling. In this regard, it is demonstrated that Fz2 is one of the genes involved in canonical Wnt signalling whose expression is increased as a result of mechanical loading both *in vivo* in mice transgenic for the high bone mass mutation G171V in *LRP5* and *in vitro* (Table 2) (65).

1.3.3. Frizzled 6 (Fz6)

Murine Fz6 expression is detected in calvaria and primary osteoblast (31). Similar to the function of murine Fz1 in Wnt/β-catenin signalling, it is shown that human Fz6 can inhibit Wnt/β-catenin signalling (66). Furthermore *in vitro* studies showed that in the presence of mineralization inducing conditions the *Fz6* expression in osteoblasts is decreased (58). These findings suggest that Fz6 can inhibit bone formation through inhibition of canonical Wnt signalling. However, the exact mechanism whereby Fz6 affects Wnt/β-catenin signalling is unknown and further studies are needed to elucidate this.

1.4. Low-density-lipoprotein receptor Related Proteins

The low-density-lipoprotein receptor related protein (LRP) family is a group of evolutionary conserved cell-surface receptors with a function in a range of cellular processes (67). Previous studies have shown that various LRPs are involved in the regulation of osteoblast function and consequently in the regulation of bone mass.

1.4.1. LRP5

Initial evidence that LRP5 and in addition the Wnt/β-catenin signalling is a major pathway in the regulation of osteoblast proliferation and differentiation, osteocyte apoptosis and bone formation came from positional cloning studies of monogenic bone disorders. In 2001 loss-of-function mutations in *LRP5* were shown to be causative for osteoporosis pseudoglioma (OPPG), a disease marked by a reduced bone mass and blindness (Table 3a) (68-71). Furthermore, gain-of-function mutations in *LRP5* gene were found to result in different high bone mass (HBM) phenotypes, all characterized by an increased cortical thickness of the long bones and the skull (the so-called craniotubular hyperostoses) (Table 3a) (72-74). All identified gain-of-function mutations are located in the first β-propeller domain of *LRP5* and subsequent functional studies showed that the mutations result in a decreased binding of the Wnt signalling inhibitors sclerostin and dickkopf 1 (Dkk1) with LRP5 (see below) (Figure 2)(75-79).

Studies with different mice models suggested that Lrp5 has a primary role in osteoblast proliferation as well as in bone matrix deposition in differentiated osteoblasts (Table 3a) (31, 80-82). Furthermore, it is more recently demonstrated that Lrp5 and Wnt/β-catenin signalling are required for osteogenesis in the response to mechanical loading (65, 83-85) which is probably most relevant in the osteocyte that transmits signals of mechanical loading to cells on the bone surface (86).

In 2008 however, Yadav *et al* surprised the bone field with their findings that Lrp5, instead of having a principal role in osteoblasts, regulates bone formation and bone mass accrual by inhibiting *tryptophan hydroxylase 1* (*TPH1*) expression and serotonin synthesis in the duodenum. By a series of innovative mouse genetic experiments, they demonstrated that circulating serotonin prevents bone formation following the binding to the 5-hydroxytryptamine (serotonin) receptor 1B (Htr1b) receptor on osteoblasts by inhibiting cAMP response binding element (CREB) expression and function, CyclineD1 (CycD1) expression and osteoblast proliferation (Table 3a) (87, 88). Loss-of-function mutations in *LRP5* will

consequently lead to increased circulating serotonin levels and a low bone mass phenotype due to a decrease in osteoblast numbers and bone formation. Otherwise, *LRP5* gain-of-function mutations shall affect bone mass by decreasing serotonin levels. This was confirmed by later studies showing that serum serotonin concentrations tend to be higher in OPPG patients and are significantly lower in subjects with HBM mutations (89, 90).

Nevertheless, the amount of already existing evidence pointing towards a direct effect of LRP5 on osteoblastic cells through Wnt/β-catenin signalling was difficult to ignore and consequently, a major effort was undertaken by Cui *et al* to explain the apparent discrepancy between these two hypotheses. They again claimed a local effect of the Wnt/β-catenin pathway in osteocytes and late-stage osteoblasts and did not find any evidence that LRP5 can regulate serotonin production in the gut at all (91). In this intriguing study, the authors found no effect on bone mass when they expressed *Lrp5* HBM alleles or inactivated WT *Lrp5* in gut cells, whereas they did observe a HBM phenotype or a decrease in bone mass when the expression was targeted to osteocytes and late osteoblasts, respectively (Table 3a). Moreover, no support could be found for *Lrp5* genotype differences in the amount of serum serotonin. This study raises questions about the effect of different promoters used to drive gene of transgenes or to localize cre recombinase for the creation of conditional knockouts and about the existing assays that measure serotonin; questions that need to be addressed by future studies (91, 92). More importantly this study re-shifted the attention towards bone mass control through modulation of Wnt/β-catenin signalling promoting the discovery of new therapies for patients with a propensity to fracture.

In addition to the evidence from monogenic diseases and mouse studies, many candidate gene and genome wide association studies showed that common variation in *LRP5* is associated with BMD and fracture risk (19, 93-97). All this evidence indicates that LRP5 has a clear function in regulating bone formation (Table 3a). However, the exact mechanism whereby LRP5 functions needs to be elucidated further.

1.4.2. LRP6

The closest homologue of *LRP5* is *LRP6* which is also been implicated to have a role in the skeleton. Both receptors share 71% amino acid identity and murine models show that they are partially functionally redundant (98-100). In contrast with *Lrp5* knockout mice which have a reduced bone mass and eye problems, global deletion of *Lrp6* in mice is not viable (Table 3a). In order to clarify the role of LRP6 and LRP5 in bone metabolism, mice lacking one allele of *Lrp6* and one or two alleles of *Lrp5* were created. $Lrp5^{-/-}$;$Lrp6^{+/-}$ mice have a reduced bone mass compared to $Lrp5^{-/-}$;$Lrp6^{+/+}$ mice and in addition they develop limb abnormalities (Table 3a) (99). These results show that both LRP5 and LRP6 have a function in bone accrual, however, their actions occur at non-redundant sites (100). In 2004, Kokubu *and colleagues* described the ringelschwanz (rs) mouse which is marked by vertebral column and neural tube malformations, oligodactyly, delayed ossification, reduced cortical thickness and bone density in metaphysis and cortical bones (101). Positional cloning demonstrated that a spontaneous mutation (R886W) in *Lrp6* caused this phenotype which is similar, although less severe, than $Lrp6^{-/-}$ which is not viable (101). Further studies in the $Lrp6^{rs/rs}$ mice demonstrated that the reduced

bone mass is rather caused by increased bone resorption than by reduced bone formation (Table 3a). The reduced bone resorption is most likely due to upregulation of *rankl* expression caused by a decreased canonical Wnt signalling in the osteoblasts although, a direct effect of Lrp6 in the osteoclasts on osteoclastogenesis and bone resorption cannot be excluded either (102).

Besides a function as co-receptor in canonical Wnt signalling, LRP6 is suggested to play another role in the anabolic effect of PTH on bone formation. Several reports already linked PTH with downstream element of Wnt signalling including β-catenin (103-105). However Wan *and colleagues* showed that PTH can activate β-catenin in a distinct manner from that of canonical Wnt signalling by binding to a receptor complex formed by LRP6 and PTH1R (106).

In addition to the results of mice and *in vitro* studies, LRP6 is also shown to play a role in regulating bone metabolism in humans. Positional cloning in a family diagnosed with early coronary artery disease, high LDL levels, high triglycerides levels, hypertension, diabetes and osteoporosis identified an autosomal dominant mutation (p.R611C) in *LRP6* as disease causing in this family (Table 3a) (107). At last, several association studies showed that common variation in *LRP6* is associated with BMD and fracture risk (Table 3a) (19, 93, 95, 97). However this could not be confirmed by some other studies.

1.4.3. LRP4

Only recently, we were able to implicate LRP4, another member of the low-density lipoprotein receptor (LDLR) family, in human bone homeostasis as well (108). A previous study of hypomorphic *Lrp4* mice (*Lrp4*^{ECD}), which lack both the transmembrane and intracellular protein domains, had already shown an increased bone turnover with decreased BMD, this in addition to growth retardation, polysyndactyly and tooth developmental abnormalities (Table 3b) (109). As a consequence, the authors suggested that Lrp4 negatively regulates bone growth by acting as an antagonist of the Wnt/β-catenin signalling pathway, probably through displacement of Lrp5/6 from Wnt's receptor complex. The inhibition of Wnt/β-catenin signalling by LRP4 was already reported as early as 2005 and was partly based on the fact that the structural organization of its extracellular domain is highly similar to that of LRP5/6 (110). In a study with hypomorphic *Lrp4* mice (*Lrp4*^{dan} and *Lrp4*^{mdig}) (Table 3b), the authors were able to show that Lrp4 can act as a receptor for both dkk1 and sclerostin *in vitro*, however, this was not studied further in detail (109).

Interestingly, at the same time, we also identified LRP4 in tandem affinity purification screen as an unbiased approach to detect interaction partners to sclerostin, an inhibitor of canonical Wnt signalling (see below). By means of different functional approaches, we subsequently found that the extracellular β-propeller structured domain of LRP4 specifically facilitates the inhibitory action of sclerostin on both Wnt/β-catenin signalling and bone mineralization *in vitro*. Very importantly, we were able to confirm these findings *in vivo* in humans by the identification of missense mutations in this domain of *LRP4*, in two isolated patients with sclerosteosis and without mutations in *SOST,* encoding sclerostin or *LRP5* (Table 3b) (108).

More recently, Li *et al* also described *LRP4* missense mutations in patients with the Cenani-Lenz syndrome, who generally present with syndactyly, synostoses and kidney hypoplasia (Table 3b) (111). Overall, these studies all clearly indicate an important and novel role for LRP4 in bone growth. However, while previous reports did not provide any link between LRP4's inhibitory action on Wnt/β-catenin signalling and other members of this pathway, we clearly demonstrated the involvement of sclerostin (108). Corresponding with a complete loss of sclerostin function, both patients had a prominent limb phenotype as the different animal models used to study (partial) *Lrp4* deficiency (110, 112-116).

As mentioned, LRP4 shares homology with LRP5/6 in its extracellular domain but unlike LRP5/6 and like other members of the LDLR family, its cytoplasmic domain also contains NPXY endocytosis signals, which have been suggested to mediate the internalization of proteins and subsequent degradation/recycling (110, 117, 118). It will be of considerable interest to study this further. For instance, it might be possible that LRP4 can also function as a clearance receptor for (secreted) signalling proteins. Similar to what has been described for Dkk proteins in the presence of Kremen, a mechanism might exist whereby LRP4 enhances the suppression of Wnt/β-catenin signalling by reducing the availability of LRP5/6 receptors through complex formation, internalization and subsequent degradation (119). The finding that LRP4 is an integral element of sclerostin inhibitory action and the future unravelling of the mechanism behind this regulation can be of great importance since it clearly opens perspectives for the design of new anabolic strategies.

Finally, common variations in *LRP4* were shown to be associated with osteoporosis related phenotypes like BMD and fracture risk in as well candidate gene associations studies as by genome wide association studies (Table 3b) (120-125). These associations again confirm the importance of LRP4 in the regulation of bone metabolism in humans.

Table: 3a: Chromosomal location (Chrom), mouse models and associated human bone related diseases for LRP5 and LRP6. Tg: transgene overexpression, CKO: conditional knockout, CKI: conditional knock in, ↑: increased, ↓: decreased, Rs: ringelschwanz, ECD: extracellular domain, BMD: bone mineral density, OPPG: osteoporosis pseudoglioma, HBM: high bone mass, ADO1: autosomal dominant osteopetrosis type 1.

LRP	Chrom	Mouse model		Associated human disease		Ref
		Model	Phenotype	Disease	Mutation / association	
LRP5	11q13.4	*Lrp5*$^{-/-}$	↓ bone mass, eye problems	HBM phenotype, ADO1, Worth disease	AD gain-of-function mutations located in the first β-propeller domain responsible for the binding of sclerostin and Dkk1	[31, 68, 71, 72, 74, 77-80, 87, 88, 93-95, 97]
		CKO 2.3Col1- Cre	Normal bone mass			
		CKO Dermo1-Cre	Normal bone mass	OPPG	Missense mutations in the ECD of LRP5	
		CKO Dmp1-Cre	↓ trabecular bone mass and cortical strength	Osteoporosis	SNPs associated with BMD and fracture risk	
		CKO Villin Cre	↓ bone mass, osteoblast numbers and bone formation rate			
		CKO Vil1-Cre	Normal bone mass			
		CKI 2.3Col1a1-Cre G171V	Normal bone mass			
		CKI Dmp1-Cre G171V or A214V	↑trabecular bone mass and bone strength			
		CKI Prx1-Cre G171V	↑ bone mass in limbs but not in vertebrae			
		CKI Villin Cre G171V	↑ bone mass and bone formation			
		Tg Rat 3.6Col1 G171V	↑ bone mass and strength			
LRP6	12p13.2	*Lrp6*$^{-/-}$	Not viable	Osteoporosis and coronary heart disease	Autosomal dominant missense mutation, p.R611C	[19, 93-95, 97, 99, 101, 102, 107]
		Lrp6$^{rs/rs}$	↓ bone mass, ↑ bone resorption			
		Lrp5$^{-/-}$; *Lrp6*$^{+/-}$	↓ bone mass	Osteoporosis	SNPs associated with BMD and fracture risk	

Table: 3b: Chromosomal location (Chrom), mouse models and associated human bone related diseases for LRP1, LRP2 LRP4 and LRP8. Tg: transgene overexpression, ↑: increased, ↓: decreased, Rs: ringelschwanz, dan: digitation anormale, mdig: malformed digits, ECD: extracellular domain, BMD: bone mineral density, BMD: bone mineral content

LRP	Chrom	Mouse model		Associated human disease		Ref
		Model	Phenotype	Disease	Mutation / association	
LRP4	11p11.2	*Lrp4*$^{-/-}$	Not viable, limb and kidney abnormalities	Sclerosteosis	Missense mutations in third β-propeller domain (ECD) of LRP4	[108, 109, 111, 125]
		Lrp4$^{dan/dan}$ and *Lrp4*$^{mdig/mdig}$	polysyndactyly	Cenani Lenz syndrome	Missense and splice site mutations in the ECD of LRP4	
		Lrp4^{ECD}	↓ BMD, ↑ bone turnover, polysyndactyly	Osteoporosis	SNPs associated with peak bone mass and fracture incidence in women	
LRP1	12q13-q14			Osteoporosis	SNPs associated with BMD and BMC in post-menopausal women	[19]
LRP2	2q24-q31	*Lrp2*$^{-/-}$	↓ bone density, bone formation defects			[130]
LRP8	1p34	*Lrp8*$^{-/-}$	No bone phenotype			[126]

1.4.4. LRP8

In addition to LRP5 and 6, LRP8 was recently identified as novel membrane associated positive regulator of canonical Wnt signalling. Using several i*n vitro* studies, Zhang *and colleagues* demonstrated that LRP8 is an important determinant of Wnt3a induced differentiation and mineralization of osteoblasts. Additional studies showed that LRP8 can interact with axin via its intracellular region. Furthermore, it was shown that depletion of *LRP8* results in decreased Wnt reporter activity as a result of a decreased β-catenin stabilization and nuclear accumulation.

Although, these *in vitro* results clearly indicate that LRP8 has a role in the regulation of osteoblast differentiation and mineralization, mice lacking *Lrp8* have no clear deficiency in bone formation (Table 3b) (126). As a consequence, more studies are needed to further investigate the role of LRP8 in bone metabolism and to investigate the mechanism whereby LRP8 modulates Wnt signalling.

1.4.5. LRP1 and 2

In contrast to the large amount of studies for LRP4/5/6; fewer studies investigated the role of LRP1 and 2 in bone metabolism. *Lrp1* knockout mice are like *Lrp6* knockout mice not viable and no other mouse models are, at this moment, available to study the function of LRP1 in bone (Table 3b) (127).However, other studies showed that *LRP1* is expressed in osteoblasts and is involved in regulating the uptake of vitamin K in chylomicron remnants (128). Vitamin K is known to stimulate osteogenic and inhibit adipogenic marrow stromal differentiation. Furthermore, *in vitro* studies showed that LRP1 can interact with human Fz1. However, the interaction between Fz1 and LRP1 does not result in activation of the canonical Wnt signalling. On the contrary, LRP1 can inhibit the activation of the canonical Wnt signalling by Wnt3a, probably by interfering with the LRP6/Fz1 complex (129). In addition, 4 SNPs in *LRP1* are shown to be associated with BMD and BMC in post-menopausal women and there are indications that they also influence bone area (Table 3b) (19).

Lrp2 deficient mice are viable and show severe vitamin D deficiency and bone disease (Table 3b) (130). Furthermore, *LRP2* is located in a QTL region linked to osteoporosis in a Chinese population (131). Unfortunately, a candidate gene association study in a Chinese population could not confirm that genetic variation in *LRP2* contributes to peak BMD variation (Table 3b) (132).

2. EXTRACELLULAR MODULATORS OF CANONICAL WNT SIGNALLING IN BONE

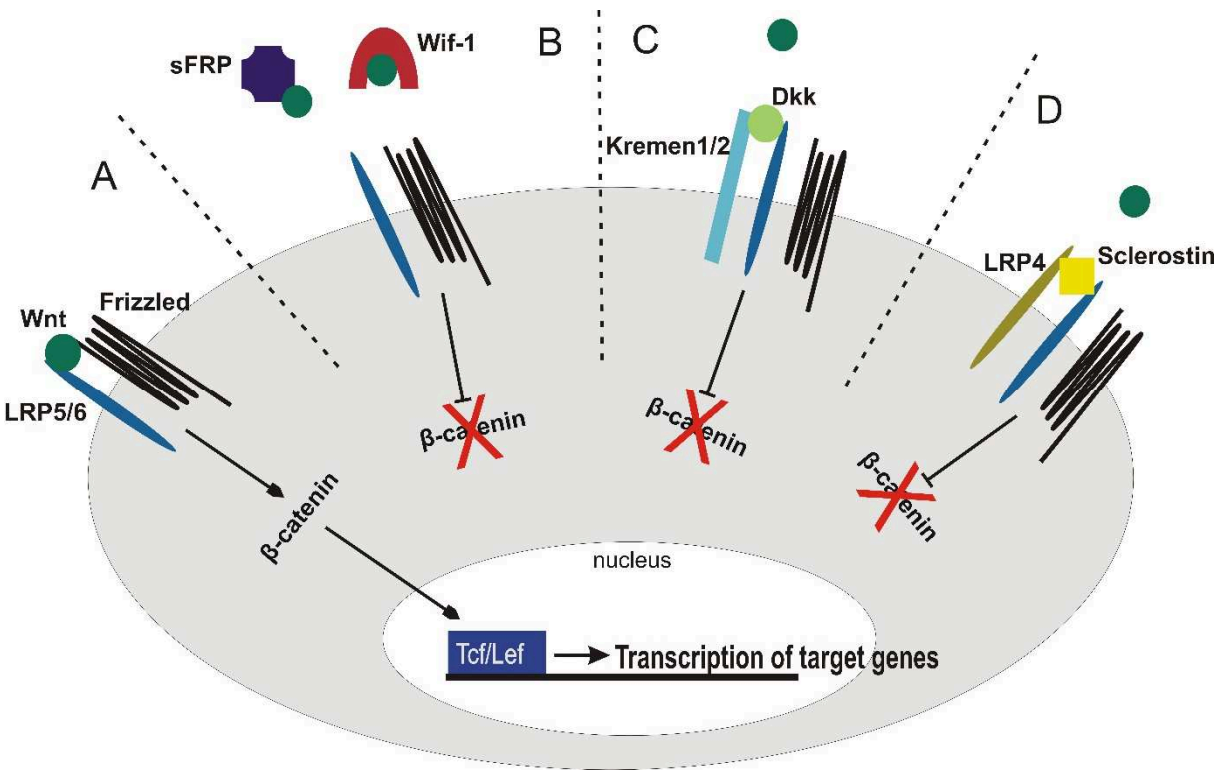

Figure 2: Extracellular modulation of the Wnt/β-catenin dependent Wnt signalling. A. Activation of the pathway by binding of Wnt on the LRP5/6-Fz receptor complex. B. Inhibition of the Wnt pathway by binding directly to Wnt ligands (sFRP family and Wif-1). C+D Inhibition of the canonical Wnt signalling by binding of Dkk or sclerostin to LRP5/6 and Kremen1/2 or LRP4, respectively.

Canonical Wnt signalling is involved in the regulation of many processes and therefore, strict modulation of this pathway is necessary. Modulation can take place as well intracellular as extracellular (133, 134). In this review we only focus on extracellular modulators of the Wnt/β-catenin signalling. Those modulators can be divided into two groups based on their mechanism of action; on one hand proteins that modulate the pathway by binding to the LRP5/6 co-receptors and on the other hand those that modulate the pathway by binding to Wnt (Figure 2). This last group can inhibit the canonical as well as the non-canonical Wnt signalling pathways.

2.1. Modulation by binding Wnt ligands

2.1.1. Secreted Frizzled related Proteins

Secreted Frizzled related proteins (sFRPs) are the first secreted modulators of Wnt signalling and they were identified more than 15 years ago (135-137). Like Wnts, sFRPs are secreted glycoproteins which structurally resemble the extracellular domain, more precisely the cystein rich domain (CRD) of Frizzled receptors known to bind Wnt ligands (135). Besides a CRD at the N-terminal site of the protein, sFRPs share a netrin-like domain at the C-terminal region of the protein. The family of sFRPs includes five members (sFRP1-5) in human which have at least partially redundant functions (138, 139). Since sFRPs bind Wnt ligands, they have the ability to inhibit both the canonical and the non-canonical pathway (Figure 2). However, the

function of sFRPs in Wnt signalling seems to be more complex than inhibition of the pathway by binding Wnt. Several studies showed that in addition to binding Wnt proteins, sFRPs can inhibit the pathway by binding Fz receptors. Furthermore, it is demonstrated that sFRPs can also stimulate the pathway depending on the context (140). Finally, it is shown that sFRPs can expand the effect region of Wnt proteins since sFRP-Wnt complex is more diffusible then Wnt ligands and therefore, have a wider distribution (140). Although the function of sFRPs in Wnt signalling is complex and more research is necessary to identify the effect of sFRPs in different processes, several studies demonstrated that all sFRPs, except sFRP5, are expressed in bone and can influence bone homeostasis.

sFRP1

The function of sFRP1 in the regulation of bone mass is already extensively studied both *in vitro* and *in vivo*. *In vitro* studies showed that sFRP1 regulates osteoblast/osteocyte differentiation, proliferation, function and apoptosis through inhibition of Wnt/β-catenin signalling as well as osteoblast induced osteoclastogenesis by binding to RANKL (141-144). An additional study showed that glucocorticoid induced bone loss caused by increased osteoblast and osteocyte apoptosis is mediated by *sFRP1* (145). Furthermore, sFRP1 is shown to regulate differentiation of mesenchymal stem cells to osteoblast or adipocytes in a dose-dependent manner. Increasing the amount of recombinant sFRP1 leads to a decrease in osteoblastogenesis and an increase in adipogenesis (146).

In vivo studies showed that disruption of the *sfrp1* gene in mice results in increased trabecular bone density in knockout compared to wild type mice. This was especially seen after 13 weeks of age when peak bone mass was reached (Table 4) (142). The increased BMD in these mice is the result of a decreased apoptosis of osteoblasts and osteocytes and of an increased osteoblast proliferation and differentiation (142). On the contrary, *sfrp1* transgenic overexpression mice show decreased bone mineral density due to reduced bone formation (Table 4) (147). Further research showed that the anabolic effect of parathyroid hormone, the only FDA approved anabolic drug so far, is blunted in *sfrp1*$^{-/-}$ mice and attenuated in overexpression mice (147, 148). As a consequence, it is suggested that anabolic effect of PTH requires the downregulation of *sfrp1* to stimulate Wnt signalling and osteoblastogenesis (147).

Finally, several association studies showed that common genetic variation in *sFRP1* is associated with bone mineral density and hip geometry parameters at different sites (19, 149-151) in Japanese and Caucasian women and in Caucasian men (Table 4). Based on these data, sFRP1 is an interesting target for the development of anabolic therapy for osteoporosis and fracture healing (148, 152).

sFRP2

sFRP2 expression was found in both mesenchymal stem cells as during in the latest stages of osteoblast differentiation (153, 154). Studies with knockout mice demonstrated that sfrp1 and sfrp2 are functionally redundant during development (155). At bird, no difference could be determined between *sfrp2* knockout mice and wild type littermates. Afterwards it was shown that these mice have brachy-syndactyly which is caused by decreased proliferation and delayed differentiation of the chondrocytes (156). These data suggest that sFRP2 is involved in the

regulation of chondrogenesis. In addition, it is demonstrated that the *sFRP2* expression is increased in multiple myeloma cells and that this increase in sFRP2 results in decreased bone formation through inhibition of the canonical Wnt signalling (157). Finally, an association study in post-menopausal women found no association between genetic variation in sFRP2 and BMD (19).

sFRP3

sFRP3 expression was first discovered in bovine articular cartilage and following studies confirmed its role in bone and cartilage inducing activities (158, 159). Since sFRP3 can bind Wnt ligands, it was suggested that it is an antagonist of Wnt signalling and as a result it was expected that treatment of osteoblast with sFRP3 would inhibit osteoblast proliferation and differentiation. However, *in vitro* studies showed that sFRP3 inhibits osteoblast differentiation but promotes osteoblast proliferation suggesting that sFRP3 can activate Wnt signalling depending on the context (159). *Sfrp3*$^{-/-}$ mice have increased cortical thickness and the anabolic response to loading is much higher in these mice compared to wild type mice (Table 4). Furthermore, it is demonstrated that these mice have increased cartilage damage when osteoarthritis (OA) was induced (160). Involvement of *sFRP3* in OA was previously shown since common variation in *sFRP3* was shown to be associated with OA in women (Table 4) (161). The exact mechanism whereby sFRP3 execute its function is unclear and need to be studied in more detailed. To conclude we can state that although, sFRP3 can influence both bone mass and cartilage, it seems that the primary function of sFRP3 is rather in cartilage than in bone.

sFRP4

The final member of the sFRP family involved in bone formation is *sFRP4*. Evidence for a role of sFRP4 in bone formation is derived from studies in mice and from linkage and association studies in humans. A first indication that *sfrp4* is an important regulator of BMD originates from studies in the senescence accelerated mouse P6 (SAMP6), a mouse model for senile osteoporosis (Table 4) (162). Using linkage and expression studies in the SAMP6 mouse Nakanishi *and colleagues* demonstrated in 2006 that increased *sfrp4* expression has an negative influence on peak BMD (162). This effect of sFRP4 on bone mass was confirmed in mice overexpressing *sfrp4*. These mice had, similar to SAMP6 mice, a decreased bone mineral density due to reduced bone formation by osteoblasts (163, 164). Using *in vitro* studies, Nakanishi *and colleagues* showed that sFRP4 can inhibit osteoblast proliferation and differentiation at least partially by suppressing the Wnt3a activity (162). In addition to the mouse models with an increased *sfrp4* expression, a *sfrp4* knockout mouse was generated by targeting exon 1. This knockout mouse showed an increased trabecular bone mass and a reduced thickness of the cortical bones (Table 4) (165).

In addition to these studies in mice, linkage and association studies in humans confirmed that sFRP4 affects bone mass. Linkage or suggestive linkage signals were achieved at chromosomal region 7p15-14 (containing *sFRP4*) with BMD at different sites in several populations with different ethnical background (166-170). Finally, both a genome wide association study and candidate gene association studies showed that several polymorphisms in *sFRP4* were

associated with bone mineral density at different site in numerous populations (Table 4) (121, 171-173).

2.1.2. Wif-1

Wnt inhibitory factor 1 (Wif1) is an evolutionary conserved protein that similar to sFRPs can bind to Wnt proteins and inhibit Wnt signalling (Figure 2). In contrast with the sFRPs, Wif1 does not contain a CRD domain but it exists of five epidermal growth factor like repeats (EGF repeats) and one Wif domain which can bind Wnt proteins (174, 175). *Wif1* expression was found *in vivo* in trabecular but not in cortical bone which implicates that *Wif1* is expressed in maturating osteoblasts (176). *In vitro* studies showed that Wif1 can interact directly with several Wnt ligands. Furthermore *Wif1* expression is increased during BMP-2 induced osteoblast differentiation in C2C12, MC3T3-E1 and KS483 cells and increased *Wif1* expression was also found in the cranial sutures of patients with craniosynostosis (154, 176-178). Additionally, *in vitro* differentiation experiments in mesenchymal stem cells demonstrated that Wif1 is a negative regulator of osteoblast differentiation and overexpression of *Wif1* stimulates adipogenesis at least partially by inhibition of canonical Wnt signalling (179). Based on these data, it is suggested that Wif1 is part of a negative feedback loop that controls osteoblast differentiation and maturation (175).

Mice completely lacking *wif1* have no skeletal abnormalities but they are more susceptible to development of radiation induced osteosarcomas (Table 4). Furthermore it was demonstrated that *Wif1* is epigenetically silenced in human osteosarcomas, suggesting that *Wif1* is a tumour suppressor gene (180, 181). Osteoblast specific *wif1* overexpression mice have, like knockout mice, no bone phenotype but *wif1* overexpression results in disrupted stem cell quiescence leading to loss of self-renewal potential of these cells (Table 4) (182).

2.1.3. Glypicans

A final family of secreted proteins that can modulate canonical Wnt signalling by binding to Wnt ligands are the glypicans, a family of glycosylphosphatodylinositol (GPI)-anchored cell-surface proteoglycans (PG). This protein family contains six members (Gpc1-6) which share a characteristic domain of 14 conserved cysteine residues (183, 184). Gpcs are regulators of ligand-receptor encounters and they can thereby control development through several signalling pathways including Wnt signalling. Depending on the cellular context Gpcs can act as inhibitors or stimulators on different pathways (183, 184).

In vitro studies showed that glypicans can stimulate Wnt/β-catenin signalling pathway by binding to both Wnt ligands and Fz receptors (185-188). Consequently, it is possible that Gpcs can stabilize the binding of Wnt with Fz. In addition, it is shown that sclerostin contains a potential binding domain for heparan sulphate proteoglycans such as glypicans which suggest that Gpcs also might be able to influence the action of sclerostin (189).

In literature, we found evidence that Gpc1, 3 and 4 are somehow involved in the regulation of bone metabolism. However, the mechanism whereby these Gpcs influence bone metabolism remains unclear. First, Gpc1 is highly expressed in the skeleton and in bone marrow (190) and it can inhibit Wnt/β-catenin signalling (191). In addition, it is shown that deletion of a

chromosomal region containing Gpc1, results in Albright hereditary osteodystrophy (AHO), a disease marked by short stature, short metacarpals, a round face and a high incidence of mental retardation (192, 193).

Like Gpc1, Gpc3 has an inhibitory effect on canonical Wnt signalling in murine cells (188). Furthermore, it is shown that Gpc3 is expressed during osteogenesis and mutations in this gene are shown to cause Simpson-Golabi-Behmel syndrome (SGBS) (194, 195). SGBS is characterized by pre- and postnatal overgrowth, visceral and skeletal anomalies and increased risked for embryonal tumours (194). In *gpc3*$^{-/-}$ mice it is demonstrated that all three Wnt signalling pathways are affected (188, 196). In addition, it is shown that besides Wnt signalling, additional pathways like the Hedgehog pathway are involved in the development of SGBS (196).

In addition to Gpc1 and 3, there are some indications that Gpc4 is also involved in bone metabolism. Deletion of *knypek* the homologue of *Gpc4* in *zebrafish* results in craniofacial skeletal defects (197, 198). Gpc4 effects bone metabolism and cartilage mainly via modulation of the Wnt/PCP pathway (186). Although, it is also shown that Gpc4 can modulate Wnt/β-catenin signalling through binding with Dkk1 (see below) (199).

The above described results clearly indicate that the glypican protein family is involved in the regulation of bone metabolism. In addition to a role in the three different Wnt signalling pathways, Gpc are also involved in the regulation of other pathways like for example the hedgehog pathway which makes it even more complex to identify the exact function of all Gpc proteins.

Table 4: Chromosomal location (Chrom), mouse models and associated bone related diseases for sFRP1-4 and Wif-1. Tg: transgene overexpression. ↑: increased, ↓: decreased, SAMP6: senescence accelerated mouse P6, BMD: bone mineral density.

	Chrom	Mouse model		Associated human bone disease		Ref
		Model	Phenotype	Phenotype	Association / mutation	
sFRP1	8p11.21	*Sfrp1*$^{-/-}$ Tg *sfrp1*	↑ Bone mass and bone formation ↓ Bone mass and bone formation	Osteoporosis	SNPs associated with BMD and hip geometry	[19, 142, 147, 150, 151]
sFRP2	4q31.3	*Sfrp2*$^{-/-}$	Brachy-syndactyly	Multiple myeloma	↑ *sFRP2* expression resulting in decreased bone formation	[156, 157]
sFRP3	2qter	*Sfrp3*$^{-/-}$	↑ Cortical thickness, ↑ response to mechanical loading and ↑cartilage damage as a consequence of induced osteoarthritis	Osteoarthritis	Associated SNPs in women	[160, 161]
sFRP4	7p14.1	SAMP6 Tg 2.3Col1a1 *sfrp4* *Sfrp4*$^{-/-}$	↓ Bone mass and bone formation, senile osteoporosis ↓Bone mass and number of osteoblasts ↑ Trabecular bone mass, ↓ thickness cortical bones	Osteoporosis	Association with BMD and hip geometry parameters	[162, 164, 168, 171-173]
Wif-1	12q14.3	*Wif-1*$^{-/-}$ Tg 2.3Col1a1 *Wif-1*	No bone phenotype, ↑ sensitivity to radiation-induced osteosarcoma formation No bone phenotype	Osteosarcoma	*Wif-1* expression epigenetically silenced	[180-182]

Table 5: Chromosomal location (Chrom), mouse models and bone disease related to sclerostin, SOST-DC1, Dkk1 and Dkk2. Tg: transgene overexpression. ↑: increased, ↓: decreased, OC: osteocalcin, APO E: Apolipoprotein E, dkk1$^{d/d}$: doubleridge mouse (hypomorphic dkk1), (p)BMD: (peak)bone mineral density

	Chrom	Mouse model		Associated human bone disease		Ref
		Model	Phenotype	Phenotype	Association / mutation	
Sclerostin	17q11.2	*Sost*$^{-/-}$	↑ bone mass and bone strength	Sclerosteosis	Loss-of-function mutations	[201, 202, 204-207, 213, 219, 220, 222]
		Tg *sost*	Osteopenia, ↓ bone mass and strength	Van Buchem disease	52kb deletion containing a Mef2c enhancer binding sequence downstream of *SOST*	
		Tg OC+APOE *sost*	Osteopenia, ↓ bone mass and strength	Osteoporosis	SNPs associated with BMD and fracture risk	
SOST-DC1	7p21.1				ions with pBMD in women	[230]
Dkk1	10q11.2	*dkk1*$^{-/-}$	Di		ions with BMD and hip geometry	[20, 237-241, 244-246]
		dkk1$^{+/-}$ and *dkk1*$^{d/d}$	↑ Bone mass and polysyndactyly	Paget's disease	Increased *Dkk1* expression in Paget's disease patients	
		Tg *dkk1*	Osteopenia, ↓ bone mass and bone formation	Multiple myeloma	Increased Dkk1 expression in patients with multiple myeloma	
Dkk2	4q25	*dkk2*$^{-/-}$	↓ bone mass and osteoblast activity	Osteoporosis	Associations with hip BMD	[19, 251]

Modulation by binding LRP5/6 co-receptors

2.1.4. SOST

Initial evidence that *SOST*, which encodes the protein sclerostin, is involved in the regulation of bone formation is delivered by the identification of disease causing mutations in patients diagnosed with sclerosteosis, a genetic condition with an autosomal recessive manner of inheritance characterized by a progressive bone overgrowth especially at the skull, mandible and tubular bones, large stature and syndactyly. As a result of the hyperostosis of the skull, sclerosteosis patient have clinical symptoms, including facial palsy, deafness and blindness due to compression of the cranial nerves (Table 5) (200-205). Subsequent to the identification of mutations in sclerostin as disease causing for sclerosteosis, a 52kb deletion downstream of *SOST* has shown to cause Van Buchem disease which highly resembles sclerosteosis however the phenotype is milder (Table 5) (206). More recent research demonstrated that the deleted region in patients with van Buchem disease contains a myocyte enhancer factor 2C (Mef2c) binding site. Deletion of this site results in decreased transcription of *SOST* (207, 208). Sclerostin is predominately expressed by osteocytes and based on its structure with a cysteine knot, it was classified in the DAN family of glycoproteins, a family of BMP antagonists (201, 209). Although sclerostin is shown to inhibit BMP-stimulated bone formation, it does not inhibit the stimulation of direct BMP target genes. Alternatively, it antagonizes canonical Wnt signalling by binding to LRP5/6 and preventing the activation by Wnt proteins (Figure 2) (210-212). This inhibitory effect of sclerostin on Wnt/β-catenin signalling is disrupted in the presence of the previously mentioned HBM-causing mutations in the first β-propeller of LRP5 which is responsible for the binding with sclerostin (76, 77).

By the identification of *SOST* as disease causing gene for sclerosteosis and Van Buchem disease, sclerostin and Wnt/β-catenin signalling became interesting targets for further research in order to identify the regulatory mechanism of bone homeostasis. In this regard mice either lacking or overexpressing sclerostin were studied. *Sost*$^{-/-}$ mice have a high bone mass phenotype with increased BMD, bone volume and bone strength due to elevated bone formation (213, 214). *Sost* overexpression mice on the contrary have reduced bone density, volume and strength due to a decreased bone formation rate (Table 5) (207, 209). Subsequently, studies investigating the anabolic effect of intermittent PTH treatment showed that the anabolic effect of PTH on bone is at least partially mediated by sclerostin since in *Sost* transgenic mice, sclerostin expression is reduced after PTH treatment while in *Sost*$^{-/-}$ mice the PTH-induced bone gain is severely diminished. Additional evidence that sclerostin is involved in the anabolic response of PTH, is demonstrated in healthy men where PTH infusion results in a decline of the serum sclerostin levels (215). These findings indicate that sclerostin is involved in the anabolic effect of PTH. However, additional protein or anabolic pathways are also affected (105, 214, 216) and additional studies defining the interaction between PTH and sclerostin in bone metabolism are needed. At last, several *in vitro* and *in vivo* studies showed that sclerostin is involved in the osteogenic response to mechanical loading since loading reduces the expression of *SOST* by the

osteocytes resulting in increased canonical Wnt signalling leading to increased bone formation (213, 217).

In addition to the involvement of *SOST* in the development of some monogenic diseases, the effect of common genetic variation on the susceptibility for osteoporosis and related fractures were studied using association studies. Several studies showed that variation in *SOST* is associated with BMD and osteoporotic fractures in several populations from different ethnical background, gender and age (Table 5) (19, 122, 124, 218-222). These associations confirm the importance of sclerostin in the regulation of bone metabolism.

2.1.5. SOST-DC1

Sost-dc1 (sclerostin domain containing factor 1) alternatively called ectodin, wise or usag-1 is a secreted protein that like sclerostin belongs to the family of DAN/Cerebus proteins (223, 224). This family of BMP antagonists contains in addition to sclerostin and sost-dc1, PRDC, gremlin, coco, Cer1 and Dan (223, 224). In addition to inhibiting the BMP pathway, Sost-dc1 is shown to have a dual role in modulating the Wnt signalling (223, 225). First, Sost-dc1 was selected from a screen designed to identify proteins which stimulate canonical Wnt signalling (225). In contrast to this study, reporter assays showed that Sost-dc1 can block the activity of Wnt1, Wnt3a and Wnt10b in vitro by binding to LRP6 and possibly LRP4 (223, 225-228). A role for Sost-dc1 in the skeleton is suggested since in osteosarcoma cells, the expression of Sost-dc1 is suppressed by Wnt10b (228, 229). Additionally, a candidate gene association study in Chinese women showed that common variation in Sost-dc1 is associated with peak bone mass (Table 5) (230). Based on these findings Sost-dc1 is an interesting protein for further research regarding its function in regulating bone mass.

2.1.6. DKK

The Dickkopf (Dkk) family encodes for secreted glycoproteins and consists of four members (Dkk1-4). All Dkk proteins have a secretion signal and two cysteine rich domains (CRD) in common. Dkk1, 2 and 4 are more related to each other than they are to Dkk3 which has besides the two CRD domains also a soggy domain N-terminal of both domains (231). Dkks can inhibit the canonical Wnt signalling by forming a complex between LRP5/6 and kremen1/2 (krm1/2) (Figure 2) (232). Furthermore, studies showed that Dkks can also inhibit the pathway in the absence of krm1/2. In addition to an antagonistic effect, Dkk2 can, depending on the context, also activate canonical Wnt signalling (231, 233). Recent studies demonstrated for three out of four Dkk proteins (Dkk1, 2, 3) a role in osteoblast function.

DKK1

Dkk1 is predominantly expressed in mature osteoblast/osteocytes which suggest that it is involved in the regulation of bone homeostasis (234). Evidence that Dkk1 is an important player in the regulation of bone formation was found in patients with high bone mass phenotype caused by gain-of function mutations in *LRP5*. These mutations inhibit the ability of Dkk1 to bind LRP5 which results in decreased inhibition of Wnt/β-catenin signalling and

increased bone formation (76). In the past years, the effect of Dkk1 on bone metabolism and disease is more extensively studied as well in mice as in humans (235, 236).

Mice completely lacking *dkk1* (*dkk1*$^{-/-}$) die shortly after birth showing severe developmental defects including head defects and limb dysmorphogenesis (237). Mice heterozygously lacking *dkk1* (*dkk1*$^{+/-}$) are characterized by an increased bone mass which is also observed in the doubleridge mouse (*dkk1*$^{d/d}$, hypomorphic *dkk1*). Both *dkk1*$^{d/d}$ and *dkk1*$^{-/-}$ mice show postaxial polysyndactyly. By studying the genetic crosses of *dkk1*$^{d/d}$ and *dkk1*$^{+/-}$ mice, it was demonstrated that the expression of *dkk1* is inversely proportional to the increase in bone mass whereby bone mass was highest in *dkk1*$^{d/-}$ mice (Table 5) (238-240). These findings were also confirmed in a *Dkk1* transgenic overexpression mouse which is marked by osteopenia due to decreased osteoblast numbers and reduced bone formation (Table 5) (239, 241). In addition to mouse models with an altered *dkk1* expression, an important role of dkk1 in bone was also demonstrated in a mouse model for post-menopausal osteoporosis (ovariectomized mouse) (242), in a model for inflammation induced bone loss (THFtg mouse) and a mouse model for steroid induced osteoporosis (243). All these mice were marked by a reduced bone mass which was increased after blocking dkk1. This indicates that the decrease in bone mass is at least partially due to inhibition of the Wnt signalling leading to reduced bone formation by Dkk1 (236, 242, 243).

In addition to the effect of *Dkk1* on bone mass found in mice, several candidate gene association studies in human were performed with BMD and bone strength parameters in order to evaluate the effect of genetic variation in *Dkk1* on susceptibility for osteoporosis (Table 5). No significant associations between SNPs in *Dkk1* and BMD were found in three different studies (19, 97, 244). However, one study showed that genetic variation in *Dkk1* was associated with the length of the hip axis which influences fracture risk (244). More recently, it was suggested that a rare variation in *Dkk1* predisposes carriers to develop primary osteoporosis. However, *in vitro* studies couldn't confirm that the variation effects Wnt signalling (20).

Furthermore the effect of Dkk1 on other diseases affecting bone such as Paget's disease of bone (PDB) and multiple myeloma (MM) was studied. Both the expression of *Dkk1* in osteoblasts and the circulating levels of *Dkk1* were increased in patients with PDB and MM (Table 5) (245, 246). The mechanism whereby Dkk1 influences Paget's disease is still unclear. It is suggested that increased expression of *Dkk1* has an influence on the initial lytic phase of the disease. In MM patients, due to the overexpression of *Dkk1* minimum or no osteoblastic activity was found (246-248). All data of mice and human studies clearly indicate that Dkk1 has an important function in regulating bone formation which makes it an interesting target for drug development (235, 236).

DKK2

In contrast with Dkk1 which is a clear antagonist of canonical Wnt signalling, the effect of Dkk2 is more complex. Depending on the cellular context Dkk2 can stimulate or inhibit the pathway, both by binding on LRP6 (249, 250). *In vitro* studies showed that Krm2 can

modulate the effect of Dkk2 to LRP6. In the absence of Kremen2, Dkk2 will activate LRP6 while in the presence of Krm2, Dkk2 is an antagonist (250). The opposite effect on osteoblast differentiation was seen in another *in vitro* study in the presence of Wnt7b. In the absence of Wnt7b, Dkk2 inhibited osteoblast differentiation while it induced terminal osteoblast differentiation in the presence of Wnt7b (251). Mice completely lacking *dkk2* have osteopenia and reduced osteoblast activity caused by a defect in terminal osteoblast differentiation and mineralization (Table 5) (249, 251, 252). *In vitro* studies in KS483 cells confirmed that in order to form mineralized bone matrix by mature osteoblast, Wnt/β-catenin signalling needs to be downregulated which is at least partially achieved by upregulation of *dkk2* (177). In humans, evidence that Dkk2 has a function in regulating bone metabolism was found by one association study which found an association between common SNPs in *Dkk2* and hip BMD in Caucasian post-menopausal women (Table 5) (19).

DKK3

The function of Dkk3 in bone metabolism is less well studied. However, there is some evidence that it is involved in the regulation of bone formation. First, *Dkk3* is highly expressed in the mesenchymal condensations during skeletal formation (253). In addition, Dkk3 is suggested to be involved in the pathogenesis of osteosarcoma since increased expression of *Dkk3* results in reduced motility and invasion of osteosarcoma cells by affecting the intracellular concentration of β-catenin (254). Finally, *in vivo* studies showed that Dkk3 has a regulatory role in enchondral bone formation since it can inhibit osteogenesis but not chondrogenesis (255).

2.1.7. Kremen

Kremen 1 and 2 (Krm1/2) are single pass trans-membrane receptors with high affinity for Dkk proteins. Krm1/2 can form a trimolecular complex with Dkk and LRP5/6 which is rapidly endocytosed resulting in inhibition of the canonical Wnt signalling (Figure 2) (119). In addition to a function as inhibitor of the Wnt/β-catenin signalling pathway, it is demonstrated that both Krms can promote the pathway by binding directly to LRP6 in absence of Dkk1 (256, 257). *Krm1*$^{-/-}$ and *krem2*$^{-/-}$ mice were viable and fertile and did not show abnormalities which indicate that Krm1 and 2 have overlapping functions (258). However, at 24 weeks of age *krm2*$^{-/-}$ mice have increased bone formation. Based on these findings different mouse models were designed. First, a *krm1* and *2* double knockout mouse was created. These double knockout mice were also viable and fertile but 74% of them had ectopic postaxial forelimb digits. Furthermore, these mice had elevated Wnt/β-catenin signalling, increased bone mass and increased bone formation rates at 12 weeks of age, 12 weeks earlier than *krm2*$^{-/-}$ mice. Limb defects similar as found in the krm double knockout mice, were previously described in *Dkk1* mutants. By creating a triple knockout mouse (*krm1*$^{-/-}$; *krm2*$^{-/-}$; *dkk1*$^{+/-}$) in which the ectopic growth of digits is enhanced, a genetic interaction between krms and dkk1 in digit development was demonstrated. Deletion of one *dkk1* copy does not result in an additional increase in bone mass compared to the bone mass of *krm* double knockout mice (258, 259).

The observation that *krm2*$^{-/-}$ mice develop a high bone mass phenotype at 24 weeks of age indicates that *krm2* is, compared to *krm1*, predominantly expressed in bone. As a consequence, a transgenic mouse overexpressing *krm2* in osteoblasts was created (259). This mouse developed a osteoporotic phenotype which was caused by both decreased bone formation and increased bone resorption, indicating that *in vivo* krm2 has a function in regulating bone formation and bone resorption, at least in mice (259).

2.1.8. R-spondins

Recently, a novel protein family has been associated with Wnt/β-catenin signaling activation and regulation. The R-spondin family of secreted Wnt agonists consists of 4 members (Rspo1-4) that share about 60% of sequence homology and common structural organization (260). It has been shown that RSPO proteins can synergize with Wnt ligands in stabilization of cytosolic β-catenin level, thus upregulating the Wnt/β-catenin signaling (261-263). Although the involvement of Rspo proteins in canonical Wnt signalling is well documented, the precise mechanism of this regulation is yet to be elucidated. Strong evidence suggests that R- spondins are capable of binding to LRP6 and thereby disrupting the LRP6/Dkk/Krm complex responsible for internalization of LRP6 (263, 264). Release of LRP6 from this inhibitory mechanism results in increased activation of canonical Wnt signaling. Some authors suggest however, that Rspo proteins can activate canonical Wnt pathway independently from the Wnt ligands (265).

More recently, three new leucine-rich repeat-containing G protein-coupled receptors (Lgr4, 5 and 6) were identified which can bind all four R-spondin proteins *in vitro* (266-268). Lgrs are 7 trans-membrane receptors with a large N-terminal extracellular domain that contains a series of leucine-rich repeats. Functional studies demonstrated that R-spondins stimulate Wnt signalling through Lgr4, 5 and 6. However the mechanism implicated in this activation is still unclear. It is suggested that the Lgr/Rspo complex physically interacts with the LRP/Wnt/Fz complex at the cell membrane since it is shown that Lgr4, 5 and 6 co-immunoprecipitate with Fz5/7 and LRP5/6 on the cell surface (261, 269, 270).

Recent data provided a link between Rspo family and bone metabolism. However, no data are available about the role of Lgr4, 5 and 6 in this process. Rspo proteins are relatively highly expressed in skeletal tissues during development and postnatally (271, 272). In addition, *rspo2* deficiency results in skeletal developmental defects in mice (272). It has also been shown that *Rspo4* mutations cause anonychia in humans (absence or severe hypoplasia of fingernails and toenails). Interestingly, similar symptoms were also observed in sclerosteosis patients (273). Furthermore a genome-wide association studies (GWAS) supplied evidence that *Rspo3* is involved in bone metabolism by demonstrating an association between common variation in *Rspo3* and BMD (19). At last, an interesting link between Rspo1 and bone formation was supplied by the research on a murine arthritis model which demonstrated that Rspo1 was able to prevent bone inflammation-related damage by the modulation the Wnt pathway and upregulation of the bone formation (274). Altogether,

these findings indicate an important role of Rspo proteins family in the control of bone metabolism.

3. CANONICAL WNT SIGNALING AS TARGET FOR DRUG DEVELOPMENT

Osteoporosis is a common disease marked by reduced bone mass and increased fracture risk. Fractures due to osteoporosis are associated with substantial pain, disability and excess mortality. Consequently, osteoporosis results in a high financial burden for society. In order to reduce the risk of fractures, first line management includes lifestyle modifications (cessation of smoking, reduction of alcohol consumption and increased physical activity) and calcium and vitamin D supplementation (275). In addition, specific osteoporosis drugs exist of which bisphosphonates, strontium ranelate, raloxifene, denosumab and parathyroid hormone peptides are currently the most important (275, 276). Except for parathyroid hormone peptides, these are all antiresorptive agents that prevent further bone loss by inhibiting the activity of osteoclasts. However, since bone resorption and bone formation are tightly coupled, they all have the important limitation that they eventually also lead to a decrease in bone formation by osteoblasts.

Therefore, much interest is currently given to bone-anabolic drugs that can increase bone mass to a greater extent than antiresorptive interventions. Moreover, they have the ability to improve bone strength in general since they can affect bone quality as well (277). To date, the only anabolic agent available is full-length parathyroid hormone (PTH 1-84) in the United States (US) or its N-terminal fragment, teriparatide (PTH 1-34) in Europe (275, 277). Because of safety concerns, duration of PTH treatment is limited to 18 and 24 months in Europe and US, respectively. Additional drawbacks are the daily subcutaneous administrations and several side-effects (277, 278). Obviously, the search for other anabolic therapies will benefit from the recent progress in the understanding of the molecular basis of osteoporosis. Signalling pathways with a primary role in osteoblast differentiation and bone formation are obviously of special interest. Wnt/β-catenin signalling has really proven its potential since its initial discovery in the bone field in 2001.

Here, we discuss some interesting targets (sclerostin, Dkk1 and sFRP1) of canonical Wnt signalling to improve bone formation and fracture healing and interesting targets to reduce bone loss during immobilization. Although some of these novel therapies already offer much promise, long-term safety of these therapies must yet be determined. This is especially important when treatment aims to increase Wnt/β-catenin signalling since unregulated activation of Wnt/β-catenin signalling is associated with cancer. Careful monitoring for skeletal and extraskeletal safety is thus obligatory when studying the long-term blockade of different Wnt antagonists.

3.1. Sclerostin antibodies

Short-term administration of a sclerostin-antibody (Scl-Ab, AMG 785 or Romosozumab) has been shown to result in marked increase in bone formation, bone mass and bone strength in both an aged ovariectomized (OVX) rat model of postmenopausal osteoporosis and using

gonad-intact female cynomolgus monkeys (279, 280). One phase-1 clinical trial in postmenopausal women showed that a single subcutaneous dose of a Scl-Ab was generally well-tolerated and increased bone formation markers and BMD of the lumbar spine and total hip (281). Noteworthy, all these studies showed an increased bone formation with either no change or a decrease in bone resorption. This has not been observed with other anabolic agents (such as PTH) and further augments the therapeutic potential of Scl-Abs (275, 281).

A phase-2 trial has been conducted to compare the efficacy of sclerostin neutralisation with alendronate (a bisphosphonate) and teriparatide (PTH) showing significant improvement in efficacy when compared to currently available treatments (282). Phase-3 trial is currently ongoing. Scl-Ab have also been tested for their potential in fracture healing. Both in the closed femoral fracture rat model and in a primate fibular osteotomy model in cynomolgus monkeys, Scl-Ab already proved to be able to significantly increase bone mass and strength at the site of fracture (283). Moreover, Scl-Ab were shown to increase the regeneration of both traumatized and un-traumatized metaphyseal bone (284). Recently, a study of mice lacking the sclerostin gene also showed accelerated bridging, greater callus formation and increased bone formation and strength in the callus of these mice (285). Two phase II clinical trials with a Scl-Ab administered to patients with acute tibia diaphyseal fractures or displaced intertrochanteric hip fractures are now on-going (286). Finally, Scl-Ab are studied for their ability to prevent bone loss during immobilization (286, 287). In a hindlimb-immobilization rat model it is already demonstrated that Scl-Ab induced increases in trabecular bone volume and thickness, bone formation rates, and mineralizing surfaces when compared to normal controls (286, 287). The results of sclerostin-based therapy are very promising and the therapy is particularly interesting because of the restricted expression pattern of the protein. However, recent studies in the field of vascular pathophysiology challenge this perception with a growing line of evidence suggesting sclerostin expression in calcifying vascular tissue (288). Morover, circulating sclerostin levels were recently associated with mortality rate in patients undergoing dialysis (289). The precise role of sclerostin in these processes remains unknown, as patients bearing inactivating mutations in the gene encoding sclerostin do not show an elevated risk of cardiovascular disease and *Sost* knockout mice shown no signs of vascular calcification (290, 291). It is clear that, with rising concerns about the sclerostin interaction with the cardiovascular system, special attention is required when evaluating the safety profile of currently developed treatments.

3.2. Dkk1 antibodies

Since Dkk1 is shown to be involved in regulating bone mass and increased expression is found in patients with either multiple myeloma or Paget's disease it is a second interesting target for treatment. With regard to the use of Dkk1 antibodies for multiple myeloma disease (MM), both a study in a severe combined immunodeficiency (SCID)-rab (rabbit) and a SCID-hu (human) murine model engrafted with primary MM cells, showed that anti-Dkk1 therapy stimulated osteoblastogenesis, inhibited osteoclastogenesis and thus resulted in the promotion of bone formation of myelomatous bone. Moreover, this also seemed to

significantly reduce tumour burden (292, 293). Similar results were obtained in mice bearing 5T2MM myeloma cells except for the fact that there was no effect on osteoclastogenesis or tumour burden here (248). The effects of neutralization of Dkk1 for osteoporosis have not been extensively studied yet, however, Glantschnig *et al* recently reported that Dkk1-antibodies could resolve the osteopenic phenotype of OVX mice and *Rhesus* monkeys (294, 295). In addition, anti-Dkk1 therapy was previously shown to prevent bone erosion in a TNF-induced rheumatoid arthritis (RA) model (296).

Some of these initial studies already showed that inhibition of Wnt/β-catenin signalling by DKK1 impedes the normal progression of fracture repair (297, 298). A subsequent study used normal mice treated with neutralizing antibodies against Dkk1 (Dkk1-Ab) and found that activation of this signalling via removal of Dkk1 indeed enhanced the repair process with significant gains in callus area, BMC, BMD and biomechanical properties (299). More recently, another study also showed this stimulatory effect of Dkk1-Ab on bone regeneration, and additionally suggested that these antibodies can improve the fixation of implants in rat cancellous (metaphyseal) bone (300). Additionally this study investigated the effect of these antibodies under different loading conditions (300). Compared to normal animals, in which the Dkk1-Ab increased bone volume by 50%, animals whose treated limb was paralysed showed a proportionally larger increase (233%). No disproportional increase in response was noted with regard to screw fixation. Finally, a very recent study demonstrated that of Dkk1-Ab treatment specifically regulates bone formation in growing rodents and upon traumatic injury (301). Compared to sclerostin the expression of Dkk1 is more ubiquitous and this will likely limit the efficacy of Dkk1-targeted therapy.

3.3. sFRP1 small molecules

A third interesting target for therapy is sFRP1. Studies regarding sFRP1 inhibition are less advanced then those targeting Dkk1 or sclerostin but one study showed that a small-molecule inhibitor of sFRP1, diphenylsulfone sulphonamide, can prevent sFRP1-mediated apoptosis of preosteocytes and stimulate bone formation *ex vivo* (302). Besides treatment with small molecules against sFRP1, one study tested a sFRP1 polyclonal antibody in the treatment for periodontitis and saw a reduction in periodontal bone loss, reduced osteoclastogenesis and decreased inflammatory cell infiltration (303). Finally, studies in *sfrp1* deficient mice have shown that diaphyseal fractures heal more quickly suggesting that additional mechanisms exist that can increase Wnt/β-catenin signalling to potentiate fracture healing (152).

Since sFRP1 can modulate all three different Wnt signalling pathways and since it has the ability to, depending on the cellular context, act as well as an agonist as an antagonist of Wnt signalling, tissue specific targeting will be important for sFRP1 therapy (278).

4. PROSPECTIVES

After the discovery that mutations in *SOST* and *LRP5* can cause monogenic bone diseases, it became clear that Wnt/β-catenin signalling is one of the major pathways involved in the regulation of bone metabolism. In the following years major research efforts were undertaken to elucidate the regulation of the pathway.

Despite this, still a lot of questions remain. One of the major issues that still needs to be elucidated is how one protein can influence different Wnt pathways and how this is regulated. Furthermore, it is still unclear whether the different Wnt pathways interact with each other and at which level this interaction takes place. In addition lots of research is necessary to investigate the effect of all possible Wnt-Frizzled interactions on the different Wnt pathways and more precisely on bone metabolism and all other processes regulated by Wnt signalling. The importance of canonical Wnt signalling in bone formation is well established. However, it remains unclear whether this pathway or one of the non-canonical pathways is also involved in the regulation of bone resorption by the osteoclast. Finally the role of canonical Wnt signalling in the pathogenesis of both monogenic sclerosing bone disorders and osteoporosis needs to be further clarified. As described above, three causative genes for craniotubular hyperostosis have been identified so far. Nevertheless, the genetic cause of the disease is still unknown in a number of patients. Since the three known causative genes *LRP5*, *LRP4* and *SOST* are all involved in canonical Wnt signalling it is likely that one or more additional members of the pathway are involved in the pathogenesis of craniotubular hyperostosis. As a consequence, identification of novel disease causing genes will result in novel insights in the regulation of canonical Wnt signalling as recently shown for *LRP4*. Besides a role in the pathogenesis of monogenic diseases, association studies demonstrated the importance of canonical Wnt signalling in the susceptibility of osteoporosis. Although, polymorphisms in several genes are shown to be associated with osteoporosis susceptibility and fracture risk, the causative variant and corresponding functional effect on Wnt signalling and bone metabolism remains unclear.

Although, much is yet to be elucidated, it is clear that the Wnt/β-catenin pathway regulates bone formation. As a consequence, several proteins of this pathway are suggested as interesting targets for the development of anabolic therapeutics for osteoporosis. Although the first results are promising it remains important to unravel more of the modulating mechanisms of the pathway as it is shown that Wnt signalling is involved in several other processes besides bone metabolism. Furthermore most proteins are members of large protein families and additionally several protein families can influence numerous signalling pathways which make it even more complex. Up- or downregulation of Wnt signalling is also shown to be important in the development of diseases such as cancer. Therefore, further *in vitro* and *in vivo* studies are needed to identify the effect of all different proteins depending on the cellular context.

Conflict of interest

All authors declare no conflicts of interest.

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OBJECTIVES

The general aim of this PhD thesis was to provide novel insights into the bone biology by the studies of rare osteochondrodysplasias. In particular, genetic and functional studies were conducted to further elucidate the involvement of LRP4 and the R-spondins in skeletal biology. Lastly, we aimed at the identification of novel points of possible therapeutic intervention to be used in future treatments of osteoporosis.

Specific aims of this PhD thesis were:

1. **Genetic and functional study of the involvement of LRP4 in bone biology.** This was performed by **mutation screening** of patients suffering from craniotubular hyperostosis. After the identification of a novel mutation in a sclerosteosis patient, detailed functional studies were conducted investigating the effects of the new and several previously known mutations in this gene on canonical Wnt signaling. These results are gathered in the third chapter of this thesis.

2. **Genetic and functional study of the involvement of R-spondins in bone biology.** To further elucidate the involvement of RSPO genes in bone biology we performed **expression studies** in differentiating osteoblasts. Moreover, mutation screening of these genes has been conducted in our population of craniotubular hyperostosis patients in search of rare variants of potentially pathogenic character. Finally, **candidate gene association study** has been conducted in stratified subpopulations of the Odense Androgene Study population to determine the impact of common genetic variation in *RSPO* genes on BMD. These findings are described in detail in the fourth chapter of this thesis.

3. **Continue the search for genetic causes of rare osteochondrodysplasias.** We perform a **mutation analysis** of the *NOG* gene in a family diagnosed with the facioaudiosymphalangism syndrome. We provide detailed clinical description of the family members and novel data on genotype-phenotype correlation in this disorder. These data are discussed in chapter five of this thesis.

Taken together we believe that studies presented in this PhD thesis will contribute to the current understanding of canonical Wnt signaling and bone biology in general. Moreover, our findings indicate LRP4 as a promising potential target for the development of future treatments of osteoporosis and other common bone phenotypes.

RESULTS

CHAPTER 3

A NOVEL DOMAIN-SPECIFIC MUTATION IN A SCLEROSTEOSIS PATIENT INDICATES A ROLE OF LRP4 AS AN ANCHOR FOR SCLEROSTIN IN HUMAN BONE

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ABSTRACT

Mutations in the *LRP4* gene, coding for a Wnt signaling co-receptor, have been found to cause several allelic conditions. Among these, two are characterized by a strong skeletal involvement, namely sclerosteosis and Cenani-Lenz syndrome. In this work, we evaluated the role of LRP4 in the pathophysiology of these diseases. First, we report a novel LRP4 mutation, leading to the substitution of arginine at position 1170 in glutamine, identified in a patient with sclerosteosis. This mutation is located in the central cavity of the third β-propeller domain, which is in line with two other sclerosteosis mutations we previously described. Reporter assays demonstrate that this mutation leads to impaired sclerostin inhibition of Wnt signaling. Moreover, we compared the effect of this novel variant to mutations causing Cenani-Lenz syndrome and show that impaired membrane trafficking of the LRP4 protein is the likely mechanism underlying Cenani-Lenz syndrome. This is in contrast to sclerosteosis mutations, previously shown to impair the binding between LRP4 and sclerostin. In addition, to better understand the biology of LRP4, we investigated the circulating sclerostin levels in the serum of a patient suffering from sclerosteosis due to a LRP4 mutation. We demonstrate that impaired sclerostin binding to the mutated LRP4 protein leads to dramatic increase in circulating sclerostin in this patient. With this study, we provide the first evidence that LRP4 is responsible for the retention of sclerostin in the bone environment in humans. These findings raise potential concerns about the utility of determining circulating sclerostin levels as a marker for other bone-related parameters. Although more studies are needed to fully understand the mechanism whereby LRP4 facilitates sclerostin action, it is clear that this protein represents a potent target for future osteoporosis therapies and an interesting alternative for the anti-sclerostin treatment currently under study.

Key words: Wnt/Beta-catenin/LRPs, Other diseases and disorders of/related to bone (sclerosteosis), Anabolics

1. INTRODUCTION

Sclerosteosis and Van Buchem disease are two closely related rare monogenic sclerosing bone disorders. Both disorders cause progressive generalized osteosclerosis, most pronounced in the skull, mandible and the tubular bones. The main clinical features are enlargement of the jaw and facial bones which can lead to secondary findings such as facial distortion, increased intracranial pressure often resulting in headaches, and entrapment of the cranial nerves. Sclerosteosis is a more severe disorder than Van Buchem disease and distinguishable from the latter by the presence of syndactyly and tall stature (1-3).

In 2001, loss-of-function mutations in the *SOST* gene were identified as disease causing for sclerosteosis in several families (4-6). Although linkage studies showed that the gene responsible for Van Buchem disease was located in the same chromosomal region (17q12-21), no mutations in *SOST* could be found (7, 8). Instead, a 52 kb deletion 35 kb downstream of the *SOST* gene was found in these patients (9-11). Subsequent studies showed that this deleted region contains a Mef2c binding element that is essential for *SOST* expression in adult bone (12). Sclerostin, the gene product of *SOST*, is an inhibitor of the canonical Wnt signalling pathway, which is activated when Wnt proteins bind to a receptor complex formed by low density lipoprotein receptor-related protein 5 or 6 (LRP5/6) and a Frizzled (Fz) receptor (13, 14). Upon pathway stimulation by Wnt, the intracellular GSK3β complex is inactivated leaving β-catenin unphosphorylated and causing it to accumulate in the cytoplasm, translocate into the nucleus and activate the transcription of target genes.

During the past years, sclerostin has been extensively studied as a target for the treatment of bone disorders with a reduced bone mass, like osteoporosis (15-17). A few years later a novel interaction partner of sclerostin, namely low density lipoprotein receptor-related protein 4 (LRP4) was identified (18, 19). The effect of LRP4 on bone formation was also confirmed when we identified disease causing mutations in two unrelated patients with sclerosteosis (19). Both mutations were located in the third β-propeller domain of LRP4 and decreased the inhibitory action of sclerostin due to impaired binding between both proteins. Studies in mice demonstrated that global deletion of *Lrp4* is not viable due to respiratory failure (20). However, in different hypomorphic *Lrp4* mouse models, polysyndactyly and other limb abnormalities were demonstrated (21). Syndactyly is also one of the clinical features seen in patients diagnosed with sclerosteosis. Further studies demonstrated that LRP4 facilitates the inhibitory actions of sclerostin on Wnt signalling. Recently, two independent studies investigated LRP4 biology *in vivo* in different mouse models (22, 23). Chang *et al.* generated mice lacking *Lrp4* in the osteoblast/osteocyte lineage and in osteocytes only. These mice presented with generalized bone overgrowth, more prominent in the strain lacking the gene in the early osteoblastic stage. The authors showed that these changes were mainly due to elevated bone formation rates. Moreover, antibodies targeting LRP4 were generated and tested for their efficacy in inducing bone formation in adult, healthy mice. Interestingly, these antibodies proved to be highly effective in stimulating new bone production in these animals, resembling the effects of well-characterized anti-sclerostin antibodies (22). In the second study, Xiong *et al.* generated two additional mouse

strains deleterious for *Lrp4* (23). The first strain, lacking the gene product in the osteoblastic lineage and the second being a global deletion of the gene rescued in the muscle tissue to prevent early lethality due to respiratory failure. Results were largely in line with the work of Chang *et al.*. However, inhibition of bone resorption was observed secondary to deficient production of RANKL. Interestingly, in both studies the authors detected a major increase in circulating sclerostin levels in transgenic animals. This suggests that, in the absence of LRP4, sclerostin is no longer retained in the bone tissue and cannot locally exert its inhibitory function on bone formation.

Mutations in *LRP4* have also been identified as causative in two other diseases. Cenani-Lenz syndrome, a rare autosomal recessive disorder, is caused by point mutations scattered across the whole protein but never localised within the third β-propeller domain (24). Patients affected by this disorder often show limb malformations, kidney abnormalities and syndactyly (25). Li *et al.* established that all identified mutations impaired the membrane trafficking of the protein, leading to its absence on the cell membrane (24). The membrane scarcity of LRP4 has been demonstrated to downregulate Wnt signaling via an unknown mechanism. Recently, two homozygous truncating mutations in that gene have been shown to underlay a prenatally lethal form of the syndrome (26).

Next to its major importance in skeletal and renal development, LRP4 has been shown to be essential for the formation of neuromuscular junctions (27). Autoantibodies against LRP4 have been discovered in patients suffering from myasthenia gravis (28-30). Up to one in 5000 individuals suffer from this disease, making it the most common neuromuscular junction pathology. Patients usually present with general muscle weakness and rapid muscular fatigue. The most common cause of this pathology (80%-85% of the cases) is the autoimmune response against postsynaptic acetylcholine receptors. It is estimated that autoantibodies against LRP4 can constitute for up to 5% of the cases. Moreover, two novel point mutations in LRP4 have been identified as the cause of related congenital myasthenic syndromes (31). Interestingly, these mutations were localized at the outer edge of the third β-propeller domain of the protein, in contrast to sclerosteosis-causing mutations clustered within the central cavity of the domain. These mutations were also shown to impair the MuSK signaling pathway in luciferase assay experiments while sclerosteosis mutations had no effect on this pathway. These results demonstrated a highly position-specific effect of the different *LRP4* mutations.

In this paper, we describe the identification of a novel *LRP4* mutation in a patient suffering from sclerosteosis and evaluate the effect of this and other mutations giving rise to skeletal phenotypes on Wnt signaling. To broaden the understanding of LRP4 biology we performed functional studies into the patophysiological mechanisms of sclerosteosis and Cenani-Lenz syndrome providing first evidence of the protein's involvement in recruiting sclerostin to the bone matrix in humans.

2. MATERIAL AND METHODS

2.1. SAMPLE PREPARATION

The samples were obtained after receipt of informed consent. Genomic DNA was extracted from peripheral blood leukocytes by standard techniques. Serum samples were obtained from blood collected into heparine and EDTA- free test tubes. Clot was allowed to form at room temperature and removed by centrifugation at 2000g (10 minutes). For mutation analysis DNA sample was obtained from the Portuguese patient. Serum sample for biochemical analysis was obtained from the Spanish patient and a group of 6 in-house controls.

2.2. MUTATION ANALYSIS

We analysed all exons of the *LRP4* gene, both exons of the *SOST* gene, the deletion causing Van Buchem disease and the enhancer region located in this deletion (NT 010783.151 position 7048084-7048324, www.NCBI.nlm.nih.gov/). Primers were designed to cover the exons and intron-exon boundaries as described previously (primer sequences are available upon request) (19). Amplification of all amplicons was performed by GoTaq DNA polymerase-mediated PCR (Promega Corporation, Madison, Wisconsin, USA). Amplification of the fragments was verified by agarose gel electrophoresis, simultaneously running a Generuler 100 bp Plus DNA Ladder (Fermentas). Afterwards, primers and unincorporated dNTPs were removed using exonuclease I (New England Biolabs, Inc, Ipswich, Massachusetts, USA) and calf intestine alkaline phosphatase (CIAP, Roche Applied Science, Hoffmann–La Roche AG, Basel, Switzerland). Finally, a sequencing reaction was performed directly on the purified fragments with the ABI 310 Genetic Analyser (AppliedBiosystems, Foster City, California, USA), using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 (Applied Biosystems, Foster City, California, USA). The BigDye XTerminator Purification Kit was used as purification method for DNA sequencing to remove unincorporated BigDye terminators.

2.3. EXPRESSION CONSTRUCTS AND IN VITRO MUTAGENESIS

An expression construct that contained the human full-length *LRP4* coding sequence (corresponding with the ENST00000378623 transcript in the Ensembl database; http://www.ensembl.org/index.html) was kindly provided to us by Michaela Kneissel (Novartis Pharma AG, Basel, CH) together with mutant human *LRP4* construct corresponding to the previously described sclerosteosis mutation p.Arg1170Trp. The novel mutation (p.Arg1170Gln) found in the proband and two known CLS mutations (p.Cys1017Arg and p.Asp529Asn) were introduced in the full-length wild type constructs using the QuickChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). Primer sequences are available upon request. The insert sequence was verified for the presence of the mutation and absence of PCR errors by direct DNA sequencing.

Remaining expression constructs were obtained as described previously (32).

2.4. LUCIFERASE REPORTER ASSAY

The human embryonic kidney (HEK) cell line 293T was grown in DMEM (Invitrogen) supplemented with FBS (10%v/v, Invitrogen). Twenty-four hours prior to transfection, cells were plated at 0,3 x 10^5 cells/well in 96-well plates. Cells were transfected using Fugene 6 (Roche Applied Science) according to the manufacturer's instructions. Wnt1-V5 (1 ng), mesdc-2 (2 ng), WT LRP5 (2 ng) pRL-TK (2.5 ng) and pGL3-OT (50 ng) constructs were cotransfected with WT or different mutant LRP4 constructs (2 ng). LRP4 plasmid with the previously reported sclerosteosis mutation (p.R1170W) was used as a control and constructs bearing CLS mutations (p.D529N and p.C1017R) together with novel SCL mutation (p.R1170Q) were used to assess their effect on sclerostin action. Depending on the experiment, different amounts of HA-mSost (20ng and 40ng) were cotransfected. When needed, empty pcDNA3.1 vector was added to make total DNA amount equal for all transfection experiments. Each transfection was carried out in triplicate and repeated independently in three separate experiments. Forty-eight hours after transfection, cells were lysed and firefly and renilla luciferase activity were measured on a Glomax Multi+ Luminometer (Turner Designs, Sunyvale, CA) using the Dual-Luciferase Reporter Assay System (Promega Corporation).

2.5. LOCALISATION STUDIES

HEK293T cells were plated out and cultured in 6-well plates at 9 x 10^5 cells/well. 24 h after plating out cells were transfected with 1µg of one of the LRP4 constructs: wild type protein or one of 4 different mutated constructs discussed above. 500 ng of empty pEGFP vector was co-transfected with every sample to serve as transfection efficiency control. Each of the transfections was carried out with the use of Fugene6 reagent with respect to the product manual. After 48 hours the cells were processed with Pierce Cell Surface Protein Isolation Kit as described in the manufacturers' protocol. In brief, surface proteins of intact cells were labeled with Sulfo-NHS-SS-Biotin, the reaction was quenched, cells lysed and labeled proteins were isolated with the use of NeutriAvidin-Agarose beads. Protein concentrations were measured with Qubit protein assay (Life technologies, Carlsbad, California, USA), samples were analysed with Western Blotting technique with the use of LRP4 antibody (Santa Cruz Biotechnology, Dallas, Texas, USA), ZIP14 antibody (Sigma) and antiGFP antibody (Sigma). Blots were digitalized and quantified with Imagequant LAS 4000 mini imager and ImageQuant TL software (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Samples were normalized to the ZIP14 signal to correct for membrane fraction isolation efficiency and for GFP signal to correct for transfection efficiency.

2.6. BIOCHEMICAL SERUM PARAMETERS

Levels of circulating sclerostin were measured using Sclerostin TECO High sensitive kit (TECOmedical, Sissach, Switzerland) according to manufacturer's protocol. Samples were measured in duplicate using Victor 2 multiplate reader (PerkinElmer, Massachusetts, USA).

Total alkaline phosphatase activity was measured on Dimension Vista 1500 instruments (Siemens Healthcare Diagnostics, Anderlecht, Belgium), using reagents of the same manufacturer.

Bone alkaline phosphatase activity was quantified by means of agarose electrophoresis with reagents from Analis (Suarlée, Belgium) (33).

Osteocalcin, carboxy-terminal collagen crosslinks (CTX) and serum type 1 procollagen (P1NP) were measured on a Modular EE instrument (Roche Diagnostics, Vilvoorde, Belgium) using reagents from the same manufacturer.

2.7. STATISTICAL ANALYSIS

Data are expressed as mean values ± SD. Comparisons between two measurements for a single experiment were performed using a Student's t-test (SPSS 20.0 software package, SPSS Inc). Values of $p<0.05$ were considered significant.

3. RESULTS

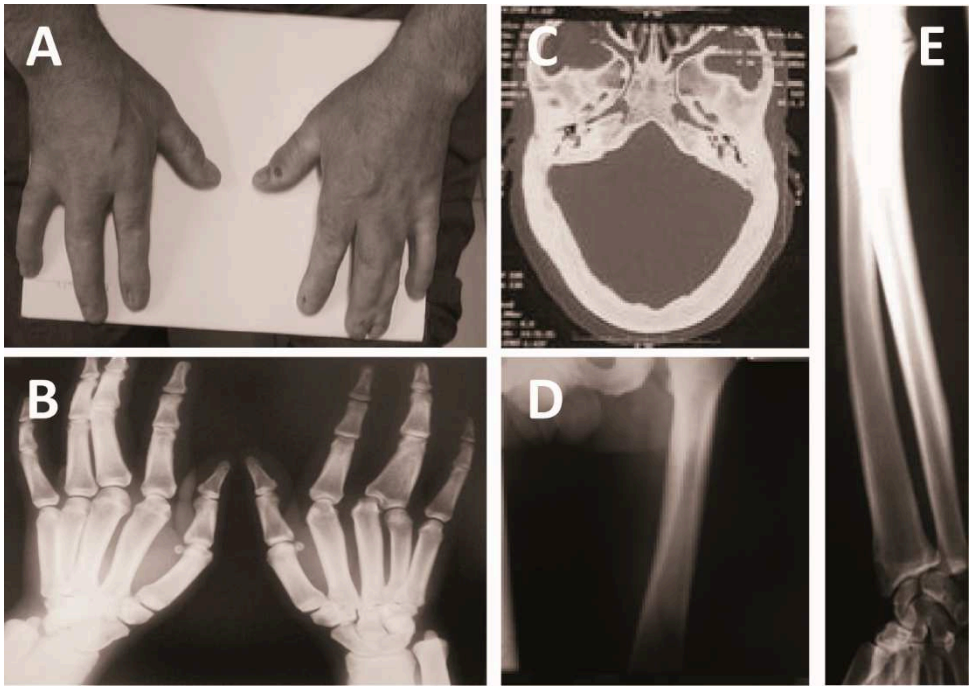

Figure 1: Clinical and radiographic features of the proband. A+B: Clinical photo and radiograph of the patient's hands. There is a complete osseous fusion of the third and fourth proximal phalanx on the left side resulting in a single digit. The third and fourth fingers on the right side show cutaneous syndactyly. C: CT scan of the skull showing a thickened calvarium. D: Radiograph showing sclerosis of the femur. E: Radiograph showing sclerosis of the radius and ulna.

3.1. CLINICAL DESCRIPTION

The proband is a 48 year-old Portuguese male born to healthy and non-consanguineous parents. He presented with typical features of sclerosteosis. His hands showed bilateral syndactyly of the third and fourth finger and bilateral nail dysplasia of the middle finger. He had a tall stature and suffered from hearing impairment. Radiographic evaluation

revealed severe sclerosis of the calvarium, base of the skull and the tubular bones. (Figure 1). Marked endosteal thickening of the diaphyseal cortex of the tubular bones was present. In addition to these typical sclerosteosis features, the patient showed hypertelorism, frontal bossing and mild asymmetry of the face.

3.2. MUTATION ANALYSIS

The patient was referred to us with a diagnosis of sclerosteosis. The known genes for this condition are *SOST* and the more recently identified *LRP4*. As a consequence, we screened both coding exons of *SOST*, checked for the presence of the 52 kb van Buchem deletion downstream of *SOST* and the complete *LRP4* gene for mutations. The Van Buchem deletion was absent in this patient and no mutations were identified in *SOST* (data not shown). No DNA was available from the parents.

In exon 26 of the *LRP4* gene, we found a homozygous mutation c.3509G>A which results in the replacement of the arginine (Arg, R) residue on position 1170 of the protein by a glutamine (Gln, Q) residue (Figure 2). The prediction programs Sift, Polyphen2 and Mutation Taster suggest that the p.R1170Q change is likely to influence the function of the protein with high respective confidence scores.

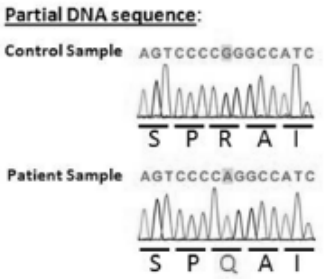

Figure 2: Partial DNA sequence chromatograms displaying the DNA sequence of the sclerosteosis patient (lower sequence) and a healthy control individual (upper sequence). We identified the homozygous missense mutation, c.3508C>A located in exon 26 of the *LRP4* gene, resulting in a p.R1170Q transition.

3.3. FUNCTIONAL EVALUATION

To evaluate the effect of the novel *LRP4* mutation p.R1170Q, on the inhibition of the canonical Wnt signalling pathway by sclerostin, either WT or mutant LRP4 were expressed in HEK293T cells together with a luciferase reporter construct. Previously reported *LRP4* mutations (p.R1170W and p.W1186S) were used as a control. Luciferase reporter assays in HEK293T cells showed that the inhibitory effect of sclerostin in the presence of the LRP4 p.R1170Q mutant is significantly reduced compared to co-transfection with wild type LRP4 (Figure 3). These results support the causative effect of the p.R1170Q mutation in this patient.

In addition, the effect of two known Cenani-Lenz syndrome mutations (p.C1017R and p.D529N) on sclerostin inhibitory action was investigated (Figure 4). The luciferase reporter

assay in HEK293T cells indicated that both mutations interfere with the inhibitory effect of sclerostin on Wnt signaling with the p.C1017R mutation being more severe in its disrupting effect. Overall however both Cenani-Lenz syndrome (CLS) causing mutations impair sensitivity towards sclerostin to a lesser extent than sclerosteosis (SCL) causing mutation.

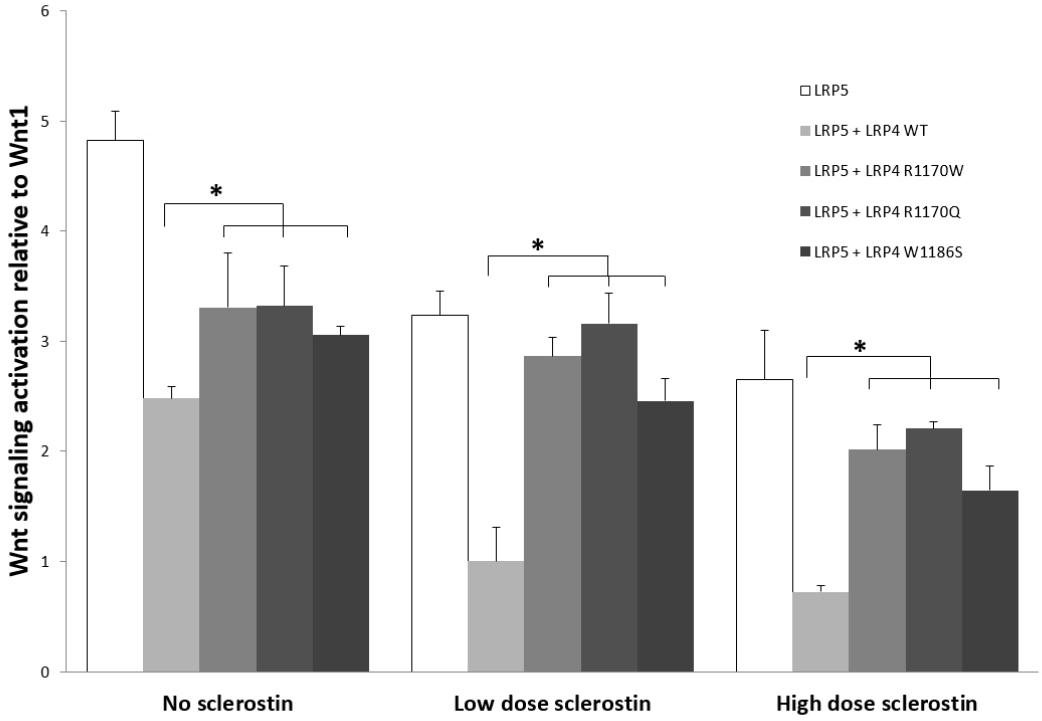

Figure 3: Functional evaluation of canonical Wnt signaling in HEK293T cells expressing transfected Wnt1 and LRP5 as well as WT or mutant LRP4 and in the presence of 0, 20 or 40 ng sclerostin. Fold induction of luciferase reporter activity in cells is normalized to the fold induction in cells expressing only the Wnt1 expression construct. Bars represent average values ± SD. *: p-value < 0.05 when compared with WT LRP4**.** Data from three independent experiments are shown.

3.4. LOCALIZATION STUDY

Membrane proteins were isolated from cells transfected with WT or mutated LRP4 constructs. Relative abundance of LRP4 on the plasma membrane was investigated. Membrane levels of LRP4 bearing sclerosteosis causing mutations were not different from those of the wild type protein (Figure 5). Both mutations underlying Cenani-Lenz syndrome displayed decreased but not abolished the protein's membrane presence. This was more prominent in the case of the p.C1017R mutation.

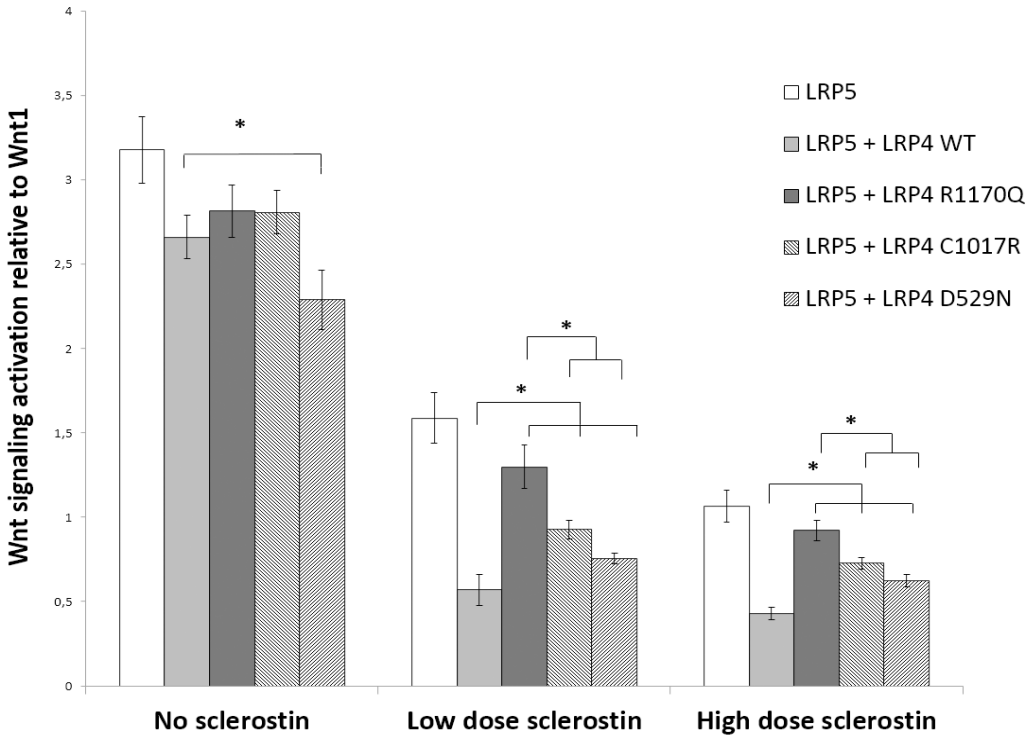

Figure 4: The effect of CLS causing mutations on sclerostin dependent Wnt signaling inhibition. HEK293T cells expressing transfected Wnt1, LRP5 and different LRP4 mutations without and with two different sclerostin concentrations (20 ng and 40 ng). Sclerosteosis causing mutation (SCL LRP4- p.R1170Q) and wild-type (WT) protein were used as controls.*: p-value < 0.05 Data from three independent experiments are shown.

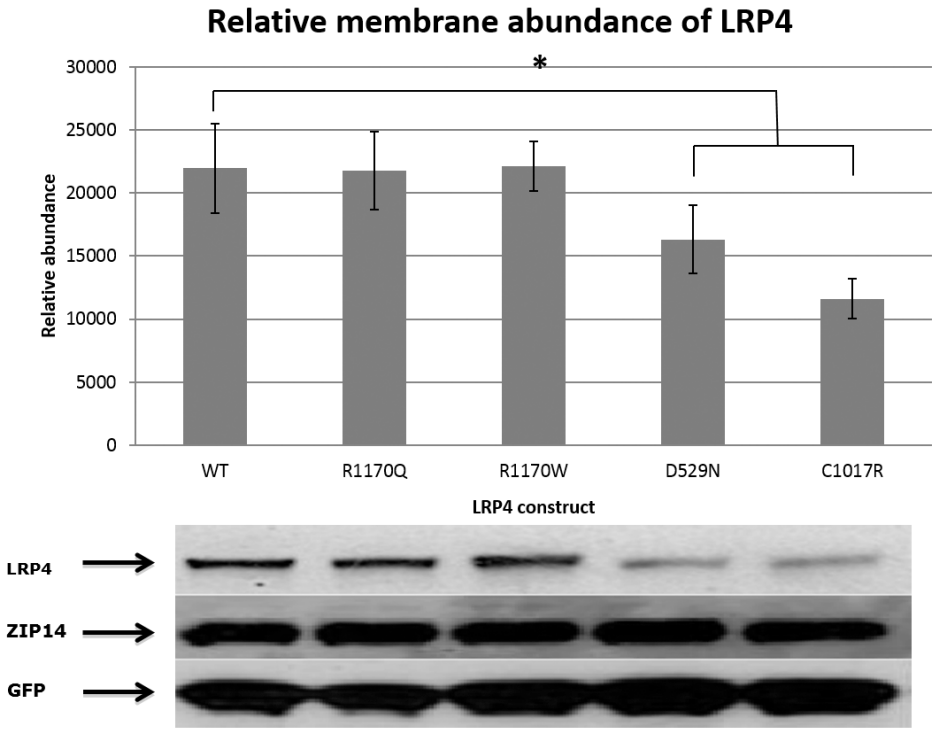

Figure 5: Relative membrane abundance of LRP4 protein bearing different mutations. Bars represent average values ± SD. Abundance is normalized against endogenous ZIP14 signal to correct for isolation efficiency and against GFP to correct for the transfection efficiency. Data from three independent experiments are summarized in the graph. *: p-value < 0.05 when compared with WT LRP4. Below: Western blot analysis of LRP4 and ZIP14 (isolation control) in membrane fractions of cells overexpressing mutated proteins and GFP in cytosolic fractions of respective cells.

3.5. BIOCHEMICAL SERUM PARAMETERS

As previous studies indicated elevated circulating sclerostin levels in mice deleterious for *LRP4* in bone cells, we investigated this parameter in a patient suffering from sclerosteosis due to *LRP4* mutation. Unfortunately no serum samples could be obtained for the novel, Portuguese patient. We therefore analysed the serum sample available from a previously described Spanish sclerosteosis patient bearing the *de novo* heterozygous p.W1186S *LRP4* mutation for circulating sclerostin levels together with 6 unrelated controls (19). The patient presented with the concentration of 1,62 ng/mL of circulating serum sclerostin. This corresponds to a Z-score of +7,92 and represents a markedly elevated level compared to the general population (according to test manufacturer's standards). All control samples presented serum sclerostin concentrations that fell within 1 SD from the mean values for respective age and gender group.

Bone formation parameters assessed in the patient's serum presented normal levels with serum osteocalcin at 17,51 ng/mL [normal range 14-46 ng/mL] and total and bone-specific alkaline phosphatase at 59 and 28,7 U/L respectively [normal ranges: 53-128 U/L and 17-48 U/L respectively]. Bone resorption was normal with carboxy-terminal collagen crosslinks (CTX) and serum type 1 procollagen (P1NP) within normal ranges at 0.195 ng/mL (normal range: <0.58) and 56.8 ng/mL (normal range: 16,3-73,9 ng/mL) respectively. All results were summarized in table 1.

Marker	Value	Normal range
Bone formation markers in serum		
Osteocalcin	17,51 ng/mL	14-46 ng/mL
Total alkaline phosphatase	59 U/L	53-128 U/L
Bone-specific alkaline phosphatase	28,7 U/L	17-48 U/L
Bone resorption markers in serum		
Carboxy-terminal collagen crosslinks	0.195 ng/mL	<0.58 ng/mL
Procollagen 1	56.8 ng/mL	16,3-73,9 ng/mL
Other serum parameters		
Sclerostin	1,62 ng/mL	0,64 ng/mL ± 0,15

Table 1: Summary of biochemical findings in the Spanish patient's serum sample.

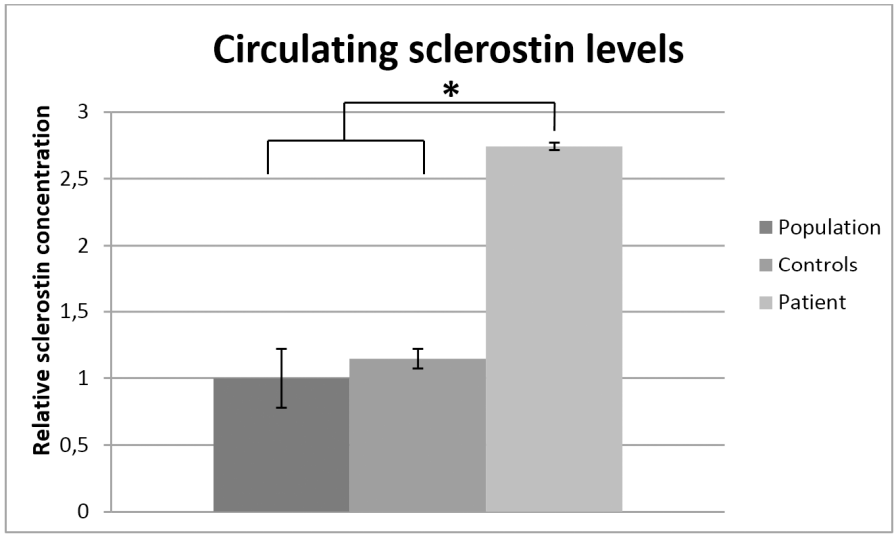

Figure 6: ELISA measurements of serum sclerostin levels. Measurement of the Spanish patient's serum sample as compared to the general population (according to the test manufacturer) and a set of in-house controls.

4. DISCUSSION

In this study, we report the identification of a novel homozygous *LRP4* mutation p.R1170Q in a patient with sclerosteosis. This residue was also affected in the previously described Greek sclerosteosis patient (19). Both patients bearing a mutation in this position present with a similar, severe clinical phenotype including syndactyly and nail dysplasia accompanied by neurological complications. In both cases hearing loss occured due to significant thickening of the calvaria and cranial nerve compression. Using dual-luciferase reporter assays, we demonstrated that this novel mutation has a similar effect on canonical Wnt signaling as previously described sclerosteosis mutations. In order to further investigate the role of LRP4 in the regulation of canonical Wnt signaling, we studied the effect of two selected Cenani-Lenz syndrome mutations. This disorder is characterized by limb and kidney malformations, radio-ulnar synostoses and syndactyly of hands and feet. In contrast with patients with sclerosteosis, no cortical thickening of the long bones was reported in these patients. None of the Cenani-Lenz syndrome causing mutations in *LRP4* are located within the central cavity of the third β-propeller domain, which is suggested to be important for the binding with sclerostin and is mutated in sclerosteosis patients. Luciferase reporter experiments demonstrated that in the presence of Cenani-Lenz syndrome *LRP4* mutations, the inhibitory capacity of sclerostin on canonical Wnt signaling is reduced, albeit to a lesser extent than in case of the sclerosteosis causing mutations. In the following localisation studies, we have demonstrated that the effect of Cenani-Lenz syndrome mutations on canonical Wnt signaling is likely due to the protein's reduced membrane presence, while sclerosteosis causing mutations in *LRP4* do not affect localization. In this regard, our findings differ from those reported by Li *et al.*, who reported complete membrane absence of *LRP4* bearing Cenani-Lenz syndrome mutations (24). In our experiments we were still able to detect mutated LRP4 in membrane fractions of HEK293T cells overexpressing the

protein. Interestingly, relative abundance of the LRP4 protein on the membrane roughly correlated with the severity of the skeletal phenotype described in patients bearing the respective mutations with p.C1017R described to underlie more severe forms of the syndrome. The observed difference might arise from the use of a different primary antibody used for the LRP4 detection. It might be that Cenani-Lenz syndrome causing mutations lead to miss-folding of the epitope detected by the antibody used by Li *et al.*, however more studies are needed to fully understand the discrepancy between both findings. In general, mutations causing Cenani-Lenz syndrome deteriorate LRP4 activity via a distinct mechanism than mutations causing sclerosteosis. In case of the novel mutation in our sclerosteosis patient, the unaffected membrane localisation suggests that impaired binding to sclerostin, like in the case of the p.R1170W mutation, is the most likely explanation of the mutation pathogenicity.

To better understand the biology of *LRP4* we obtained a serum sample from one of the previously described patients with a p.W1186S mutation in the *LRP4* coding sequence. This mutation was found *de novo* in heterozygous state but has been proven to lead to the same pathogenic effect (19). Unfortunately, the other patients were not interested in further collaboration. Interestingly, significantly elevated levels of circulating sclerostin were detected supporting the findings from recent Lrp4 mouse studies. These data indicate that the LRP4 protein on the membrane of osteoblasts has an important role in the regulation of bone homeostasis by binding sclerostin and retaining it within the bone environment. In light of these findings, serum sclerostin levels should be approached with caution when used as predictors of other phenotypes. Interestingly, bone formation and resorption markers were normal in our patient suggesting that changes in bone density observed in sclerosteosis are likely to occur due to lifelong but relatively small imbalance between these parameters. These results are largely in line with the observations from the mouse model generated by Chang *et al*. and confirm that diminishing the bone specific activity of LRP4 does not influence resorption parameters in humans. These observations remains in contrast with the findings of Xiong *et al*. (22,34).

In conclusion, the identification of a novel mutation confirms the key involvement of LRP4 in the regulation of bone metabolism. Although we are yet to understand the exact mechanism whereby LRP4 facilitates the inhibitory actions of sclerostin on Wnt/β-catenin signalling, the retention of this inhibitory protein in bone matrix and osteoblastic environment emerges as a potential explanation of this process. It is clear that LRP4 represents a potent target for future osteoporosis treatment strategies. Based on our data, we suggest that the central cavity of the third β-propeller domain is the most promising target for such treatments.

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6. AUTHORS CONTRIBUTIONS

Study design: IF,EB,WVH. Study conduct: IF,EG,ES,EB,VVH. Recruitment, sample collection and description of the patients: AMF, FJ. Data interpretation: IF,EB,WVH,GM. Drafting manuscript: IF. Revising manuscript: all authors. Approving manuscript for publication: all authors. IF takes responsibility for the integrity of the data analysis.

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CHAPTER 4

ABSENCE OF FURTHER SUPPORT FOR AN INFLUENCE OF GENETIC VARIANTS IN THE R-SPONDIN GENES ON BONE MINERAL DENSITY.

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Abstract

The R-spondins are a family of four secreted agonists of the Wnt signalling, a pathway crucial for regulation of bone formation. Over the last years, both *in vitro* studies and *in vivo* murine models shown the major importance of these proteins in the skeletal biology. Moreover, genetic variation in the *RSPO3* gene has been associated with BMD in large scale GWAS study.

The aim of this study was to further study the genetic and functional involvement of the R-spondins in bone biology. To prioritize genes for genetic screening, we performed an expression study in differentiating osteoblasts showing that *RSPO4* is not expressed under such conditions. After prioritizing *RSPO1-3* for genetic testing, we conducted a candidate gene mutation screening in a cohort of patients suffering from craniotubular hyperostosis in search for rare, activating mutations in these patients. Unfortunately, no potentially pathogenic variants were detected. To further study the relation between common variants in the R-spondins genes and BMD we decided to perform a candidate gene association study in the subpopulations of the Odense Androgen Study (OAS) stratified based on BMD. This study was performed to confirm the association signals detected in GWAS and search for causal variants with increased resolution. Unfortunately, no significant associations were detected in our cohort. Interestingly, one of the polymorphisms, namely rs140821794 (p.Met16Val) was only detected in a single individual out of the low BMD OAS subpopulation. We performed a dual luciferase reporter assay to test the functional relevance of this variant but could not confirm an effect of this polymorphism on the pathway activation.

Taken together, our data suggest that despite robust expression during bone formation and clear Wnt signaling activation in the presence of the R-spondins, the genetic variation in these genes does not contribute greatly to the genetic determination of bone mass. These findings do not diminish the potential utility of the R-spondins as drug targets modulating Wnt signaling pathway in future treatments of osteoporosis.

Introduction

The R-spondins are a family of four small, secreted agonists of Wnt signaling. The members of the family show up to 60% similarity in their pairwise amino acid sequence and homologues were described in all vertebrates and many invertebrates. All family members show a similar protein structure with an N-teminal signal responsible for secretion followed by two cysteine-rich furin-like domains, a thrombospondin type 1 repeat and a positively charged C-terminus (1, 2). Despite the fact that R-spondin 3 was initially discovered in the early 70s as a thrombin sensitive protein, it wasn't until 2011 that the specific receptor facilitating their action was discovered and light was shed on the proteins mode of action (3-5). Several groups demonstrated that Lgr 5 and Lgr4, members of the family of G-protein coupled receptors indeed facilitate the action of R-spondins and drive their activity as positive modulators of the Wnt signalling (6, 7). It has been proposed that the modulation of Wnt signaling by R-spondins occurs by binding and inactivating two ubiquitin ligases, namely ZNRF3 and RNF43 that facilitate the membrane clearance of the Wnt receptor complex composed of a Fzd receptor and a LRP5/6 coreceptor (8, 9). In this way, R-spondins increase the availability of functional receptors for WNT binding and potentiate the subsequent activation of the downstream pathway.

The importance of R-spondins in human biology has been largely correlated with early development. A number of informative mouse models have been developed and characterized to demonstrate this association. *Rspo1* null mice display impaired fertility due to aggravated gonad formation (10). In case of full Rspo2 knock-out, severe malformations were found in limbs, craniofacial bones, kidneys and lungs of transgenic mice leading to perinatal death (11). Similarly, global deletion of *Rspo3* is prenatally lethal due to abnormal placenta formation and lack of vascularisation (12). Loss of RSPO4 function is primarily known to give rise to anonychia or severe nail dysplasia (13). Next to their major involvement in the developmental processes, the R-spondins have been shown to play an important role in the skeleton. Despite the fact that *Rspo2* deleterious mice were the only ones to display a clear skeletal phenotype, the crossing of *Rspo2* $^{-/-}$ mice with *Rspo3* $^{-/-}$ animals resulted in exacerbation of the phenotype suggesting certain degree of redundancy (14). Moreover, expression of all R-spondins have been detected in limb bud and craniofacial bones (11). To add to this, common variation in RSPO3 has recently been associated with bone mineral density (BMD) in large scale GWAS studies (15). This, taken together with the important role of the Wnt signaling in bone formation, suggest that R-spondins might be important players in the maintenance of the adult bone mass.

In this work, we investigated the expression of all four R-spondins during the differentiation of KS483 mesenchymal stem cells towards osteoblasts. These cells have been proven to represent a good osteoblastic model due to their ability to produce and mineralize bone matrix under the differentiating conditions.

Moreover, we evaluate this hypothesis by performing mutation screening in a population of patients suffering from craniotubular hyperostosis. In search of rare genetic variation, we evaluate whether point mutations in *RSPO1*, *2* and 3 genes can cause this severe bone phenotype by abnormal activation of Wnt signaling. Causality of a mutation detected in this cohort is a strong indication of its involvement in the regulation of bone mass and skeletal health as proven by the identification of mutations in *LRP5*, *LRP4* and *SOST* discovered in a similar setting.

In the second part of this study, we perform an association study between common variants in *RSPO1,2* and *3* genes and BMD, in a stratified subset of the Odense Androgen Study (OAS) population. In this setup two groups of healthy individuals were selected displaying top and bottom 10% of BMD values discovered in the population and genotyping of the selected genes was performed. Obtained genotype frequencies were compared between the two groups to assess if common variation in these genes correlates with BMD.

Materials and methods

Expression studies in differentiating KS483 cells

In order to prioritize the genes for genetic analysis, we evaluated the expression of all R-spondins in maturating osteoblasts. KS483, murine, mesenchymal stem cells, kindly provided by Percuros (Leiden, The Netherlands) were differentiated towards osteoblasts for 28 days in three biological replicates. Culture medium was supplemented with ascorbic acid (at day 4 and 7) and β-glycerolphosphate (day 11 onward) as previously described. Total cellular RNA was isolated at 8 time points (d4, d7, d11, d14, d18, d21, d25, d28) during this process and cDNA for qPCR analysis was obtained using Super Script III kit (Fisher Scientific, Breda, The Netherlands).
Primers for all genes were designed with the help of the Quant Prime online tool (primer sequences available upon request). Expression of all R-spondins was assessed together with collagen 1a1, bone specific alkaline phosphatase, bone sialoprotein and osteocalcin as markers of differentiation. Beta 2 Microglobuline, Ribosomal Protein 13a and Ubiquitin C were used as reference genes. All reactions were run using Takyon SYBR green qPCR kit (Eurogentec, Seraing, Belgium) according to manufacturer's protocol on Light Cycler 480 (Roche, Basel, Switzerland). All data were analysed using qbase+ software (Biogazelle, Ghent, Belgium).

Study populations and DNA isolation

Mutation screening has been conducted in a set of 52 probands reffered to us from genetic centers from all over the world, suffering from different forms of craniotubular hyperostosis without known genetic cause (16). The patient population included individuals of different ethnical backgrounds, all displaying a selection of typical features of the disease, namely sclerosis of the skull and of the tubular bones. Additional features discovered in some patients were facial dysmorphisms with wide nasal bridge and overgrowth of the mandibular

bones, syndactyly and dysplastic nails. In some cases additional neurological complications were found including hearing loss, facial paralysis and headache due to increased intracranial pressure. All patients were screened, with a negative result, for mutations in known genes causing these phenotypes, namely *LRP4*, *SOST*, exons 2-5 of the *LRP5* gene. Furthermore the presence of the Van Buchem disease causing deletion including a regulatory element that modulates *SOST* expression was excluded.

For the association studies, two subpopulations of young, healthy men were selected from the Odense Androgen Study (OAS) (17). OAS is a population-based, observational study focused on the investigation of correlations between the endocrine status and well-defined phenotypic variables including muscle function, bone metrics and body composition in Danish men. The population consists of 783 Danish men aged between 20 and 29 years. Details on inclusion criteria and phenotyping procedures were described elsewhere (17). In short, BMD was measured at the femoral neck and the lumbar spine by dual energy X-ray absorptiometry (DXA) with the use of Hologic 4500 device (Hologic, Waltham, MA, USA). For this study, a stratified subset of individuals from the population was selected. 128 individuals with the most extreme BMD values were divided into two groups: 64 individuals with BMD T-score <-1.37 (extreme 10% of low BMD patients) and 64 individuals with BMD T-score >1.52 (extreme 10% of high BMD patients) as previously described (18).

DNA was isolated from peripheral blood leukocytes with standard techniques. Informed consent forms were collected from all participants prior to the study.

Genetic screening

Primers were designed to cover all coding exons and exon-intron boundries of *RSPO1,2* and *3* genes (primer sequences available upon request). Amplification was performed with the use of GoTaq DNA polymerase (Promega Corporation, Madison, Wisconsin, USA). Obtained products were verified by agarose gel electrophoresis, as compared to Generuler 100bp Plus DNA Ladder (Fermentas). Reactions were purified with exonuclease I (New England Biolabs, Ipswich, Massachusetts, USA) and calf intestine alkaline phosphatase (Roche Applied Science, Hoffmann-La Roche AG, Basel, Switzerland) in order to remove unincorporated dNTPs and primers. Purified DNA was used for sequencing reactions with the use of ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems, Foster City, California, USA). BigDye XTerminator Kit was used to remove unincorporated BigDye terminators and samples were run on ABI 310 Genetic Analyser (Applied Biosystems, Foster City, California, USA).

Unknown genetic variants identified in this study were tested with the use of PolyPhen 2, SIFT and mutation taster prediction programs for indication of their potential pathogenic effect on protein structure and function. Moreover, intronic variations were tested with the use of Netgene 2 and Spliceport prediction programs.

Luciferase reporter assays

Human embryonic kidney (HEK 293T) cells were cultured in DMEM medium with 10% FBS (Invitrogen). Cells were plated out at 0,3 x 10^5 cells/well in 96-well plates. After allowing 24 hours for proliferation the cells were transfected with Fugene 6 (HEK 293T) or Viafect (Saos-2) according to manufacturer's protocol (Promega Corporation, Madison, Wisconsin, USA). For the transfection of HEK293T cells Wnt1-V5 (1 ng), mesdc-2 (2 ng), LRP5 (2 ng), LRP6 (2ng), pRL-TK (2.5 ng) and TopFlash (20 ng) constructs were cotransfected with RSPO1,2 or 3 (2ng) in different configurations. Empty pcDNA3.1 vector was used to equilibrate the total amounts of transfected DNA between different samples. 48 hours post transfection the cells were lysed and luciferase activity was measured using Dual Luciferase Reporter Assay System (Promega Corporation, Madison, Wisconsin) on a Glomax Multi+ luminometer (Turner Designs, Sunnyvale, CA, USA). The ratios between firefly (TopFlash) and *Renilla* (pRL-TK) luciferase represent the relative activation of canonical Wnt signaling in investigated cells.

Results

Expression study during osteoblastic differentiation

R-spondins 1, 2 and 3 were expressed at detectable levels during the differentiation of KS483 cells. R-spondin 1 was predominantly produced during the matrix mineralization phase peaking between day 18 and day 25 of the osteoblastic maturation process. R-spondins 2 and 3 follow a common expression pattern peeking in the matrix formation phase (between day 7 and 11) and remaining relatively stable throughout the entire differentiation process. R-spondin 4 expression was not detected at any of the timepoints. Detailed data is presented in figure 1.

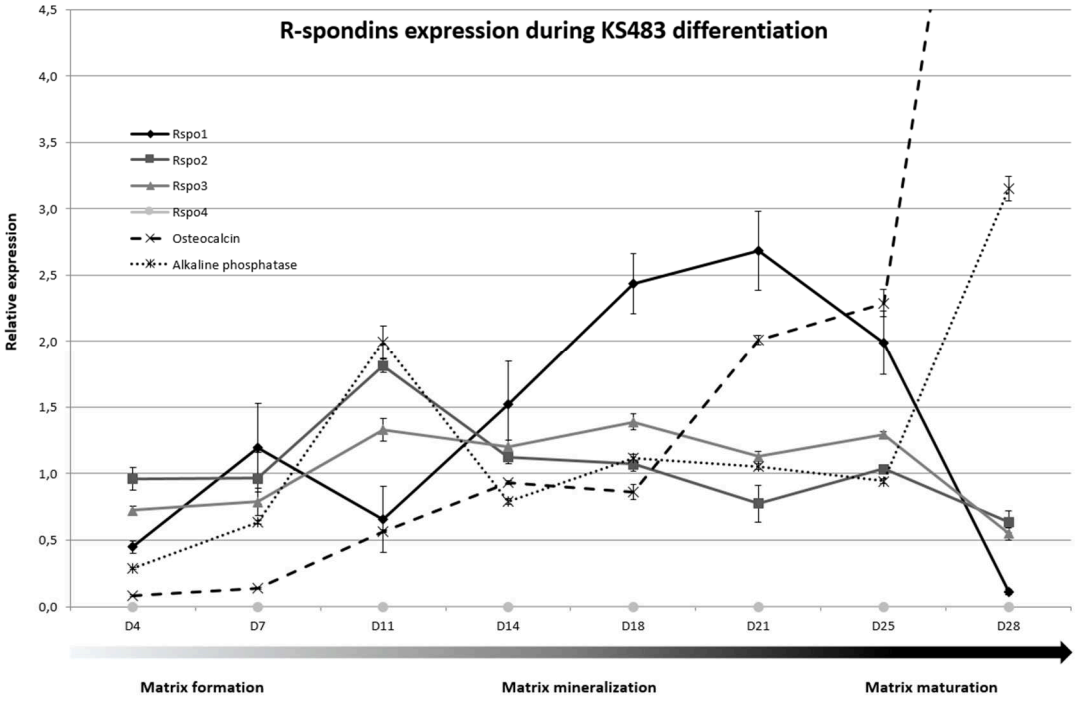

Figure 1

Expression of bone specific alkaline phosphatase, osteocalcin and *R-spondin 1,2* and *3* during osteoblast differentiation.

Mutation analysis in patients with craniotubular hyperostosis

Mutation analysis in our cohort with craniotubular hyperostosis did not reveal any novel coding variants in *RSPO1,2* and *3*. However, we identified 4 rare intronic variants (Table 1). To evaluate the effect of the rare variants, we tested them in two different prediction programs but no effect on splicing of the mRNA was predicted.

Gene	Location On the transcript	Variation
RSPO1	Intron 1	Ex1 +13 G/G->G/A
	Intron 5	Ex5 +15 G/G->G/C
		Ex5 +19 T/T ->T/C
RSPO2	Intron 3	Ex3 +21 A/A->A/G

Table 1

Summary of rare variants identified in the population of craniotubular hyperostosis, each detected in a single patient.

Association of common genetic variation in *RSPO1,2* and *3* genes with total body BMD

The genotyping of *RSPO1*, *2* and *3* in the stratified cohorts of the OAS population revealed a number of variants commonly occurring in investigated individuals (Table 2). However, none of the detected common variants shows statistically significant association with BMD in our cohort. Noteworthy, rs3734626 shows no association with BMD in OAS subpopulations. According to CEU population in HapMap, this polymorphism is in 100% LD with the GWAS tag-SNP (rs13204965) that was shown to be associated with BMD by Estrada *et al.*. All results are collected in Table 2. Interestingly, one of the coding polymorphisms located in *RSPO3* gene, namely rs140821794 (MAF=0.0233%, p.Met16Val), was only found in one individual of the low BMD subpopulation. This variant was predicted to be benign by Polyphen and Mutation taster, but SIFT indicated possible deteriorating effect on protein function. We selected this polymorphism for further functional study.

Variant	RefSNP number	Genotype	Genotypes frequencies in OAS subpopulations		*p*-value
			T-score >1.52	T-score <- 1.37	
Intron 1 RSPO1	rs41267327	CC	0,95	0,91	0,116
		CG	0,05	0,09	
		GG	0,00	0,00	
Intron 2 RSPO1	rs45577433	GG	0,89	0,88	0,784
		GC	0,11	0,13	
		CC	0,00	0,00	
Intron 2 RSPO1	rs12046650	CC	0,56	0,56	0,468
		CT	0,34	0,41	
		TT	0,09	0,03	
Intron 2 RSPO1	rs12039431	GG	0,56	0,56	0,468
		GA	0,34	0,41	
		AA	0,09	0,03	
Intron 3 RSPO1	rs11588571	CC	0,89	0,88	0,784
		CA	0,11	0,13	
		AA	0,00	0,00	
Exon 4 RSPO1 (p.K162Q)	rs36043533	TT	0,89	0,89	0,975
		TG	0,11	0,11	
		GG	0,00	0,00	
Intron 5 RSPO1	rs55852308	AA	0,58	0,53	0,941
		AT	0,33	0,42	
		TT	0,09	0,05	
Intron 1 RSPO2	rs10955475	CC	0,64	0,62	0,761
		CT	0,31	0,33	
		TT	0,05	0,05	
5' UTR RSPO2	rs716149	GG	0,72	0,73	0,957
		GA	0,28	0,27	
		AA	0,00	0,00	
Intron 1 RSPO2	rs1369068	CC	0,31	0,17	0,090
		CT	0,38	0,41	
		TT	0,31	0,42	
Exon 1 RSPO3 (p.M16V)	rs140821794	AA	1,00	0,98	0,321
		AG	0,00	0,02	
		GG	0,00	0,00	
Intron 2 RSPO3	rs2503112	AA	0,92	0,95	0,467
		AG	0,08	0,05	
		GG	0,00	0,00	
Intron 2 RSPO3	rs2503113	AA	0,98	0,98	1,000
		AG	0,02	0,02	

		GG	0,00	0,00	
Intron 3 RSPO3	rs9491699	CC	0,25	0,25	0,462
		CT	0,53	0,44	
		TT	0,22	0,31	
Intron 3 RSPO3	rs3734626	TT	0,42	0,34	0,601
		TC	0,25	0,33	
		CC	0,33	0,33	
Intron 4 RSPO3	rs41285262	GG	0,76	0,62	0,078
		GA	0,23	0,33	
		AA	0,02	0,05	
Exon 4 RSPO3 (p.L189L)	rs1892172	GG	0,18	0,22	0,917
		GA	0,50	0,40	
		AA	0,32	0,38	
Intron 4 RSPO3	rs2489629	TT	0,18	0,21	0,918
		TC	0,48	0,41	
		CC	0,34	0,38	

Table 2

Summary of genotype frequencies of all common variants identified in this study together with the association outcome (p-value).

Functional evaluation of identified variation

The functional relevance of rs140821794 (p.Met16Val) identified in one individual within the low bone density OAS subpopulation was tested with a reporter assay (Figure 2). There was no detectable difference in the RSPO3 ability to potentiate canonical Wnt signaling between wild type and polymorphism-bearing constructs. The same was true when Wnt1 was simultaneously overexpressed and when a LRP5 construct was cotransfected.

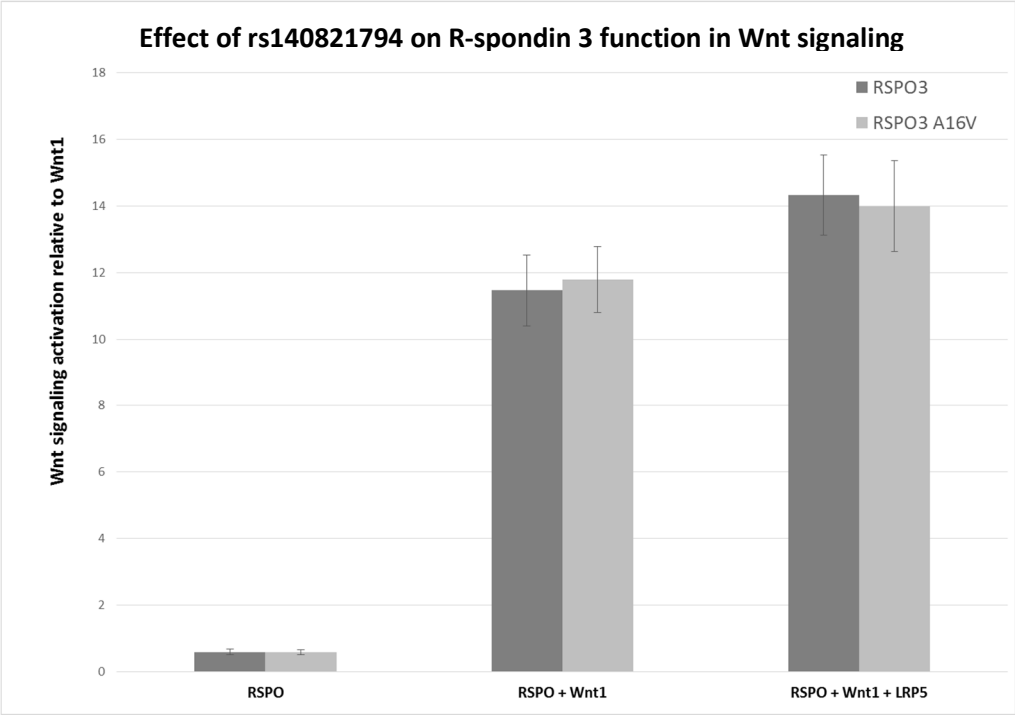

Figure 2

Functional evaluation of canonical Wnt signaling in HEK293T cells expressing transfected Wnt1 and LRP5 as well as WT or mutated R-spondin 3. Luciferase reporter activity in the cells is normalized to the induction observed in cells overexpressing Wnt1 alone. Bars represent average values ± SD.

Discussion

Numerous studies demonstrated that genes involved in the canonical Wnt signaling pathway contribute to the susceptibility for osteoporosis and fragility fractures. The identification of *LRP5*, *SOST* and *LRP4* as disease causing genes for different sclerosing bone dysplasias by us and others resulted in new insights into the genetic factors regulating bone metabolism and health of the adult skeleton (19-24). In addition, these identifications led to the development of new therapies for osteoporosis and other bone diseases. The antibodies against sclerostin, currently developed by several companies are the best example of the usefulness of the presented cohort in highlighting novel players in bone metabolism (25). In this study, we used the same approach to evaluate the *RSPO1, 2* and *3* genes, encoding the extracellular agonists of the canonical Wnt signaling pathway.

Unfortunately, in this study we could not identify any possible disease causing mutations in the *RSPO1, 2* and *3* genes. Based on our data, we conclude that mutations in coding regions of *R-spondin 1,2* and *3* genes are not a common cause of craniotubular hyperostosis in humans. However, we cannot exclude that large duplications or mutations in non-coding regulatory regions of either *RSPO1, 2* or *3* could potentially lie at the basis of these disorders.

The association studies into the effect of the common variation in these genes on BMD performed in high and low BMD cohorts from OAS population did not reveal any statistically significant signals associations. Despite the fact that the *RSPO3* tag SNP associated with BMD in large-scale GWAS studies was not included in our analysis, we genotyped a variation remaining in complete LD with this polymorphism. We were not able to confirm the association. This may indicate that common variation in *RSPO1,2* and *3* genes does not contribute to genetic determination of bone mass in the OAS population or contributes to it at a level not detectable with the power of our study. The power to detect functionally relevant common variants by the approach used in this study has been proven by the recent discovery of the expression-regulating polymorphism in *Wnt16* gene using the same setup (18). Interestingly, one of the polymorphisms in exon 1 of the *RSPO3* gene, namely rs140821794 was only detected in a single member of the low bone density cohort of the OAS population. This coding variant affects the amino acid sequence by substituting methionine at position 16 by valine and was discovered in an individual with a T-score of -1,5. According to PolyPhen-2 and mutation taster the variant is not likely to be of functional relevance, but SIFT indicates possible deteriorating effect on the function of the protein. We decided to investigate this variant further due to the ambiguity of the prediction programs when it comes to assessing its pathogenicity. The variant shows no effect on the protein's ability to potentiate canonical Wnt signaling when coexpressed with Wnt1 or Wnt1 and LRP5 coreceptor. These findings suggest that the polymorphism does not functionally affect the R-spondin 3 activity within Wnt signaling.

Although our data suggest that, R-spondins are expressed during bone formation and activate Wnt signaling, neither common, nor rare genetic variation in *R-spondin 1,2* and *3* genes contribute greatly to the genetic determination of skeletal health. These findings do not exclude the potential utility of R-spondins as druggable modulators of the canonical Wnt signaling pathway but, together with the severe phenotypes of knock-out animals, might indicate that the fundamental role of these proteins is to control skeletal development rather than bone mass maintenance.

Acknowledgements

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CHAPTER 5

FURTHER DELINEATION OF FACIOAUDIOSYMPHALANGISM SYNDROME. DESCRIPTION OF A FAMILY WITH A NOVEL *NOG* MUTATION, HYPERMETROPIA BUT NO LOSS OF HEARING.

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ABSTRACT

Mutations in the *NOG* gene give rise to a wide range of clinical phenotypes. Noggin, the protein encoded by this gene is a secreted modulator of multiple pathways involved in both bone and joint development. Proximal symphalangism is commonly observed in patients bearing mutations in this gene, however secondary symptomes are often found including typical facies with hemicylindrical nose with bulbous tip, hyperopia, reduced mobility of multiple joints, hearing loss due to stapes fixation and recurrent pain from affected joints. With large variation of the phenotype both within and between affected families careful delineation of the genotype-phenotype correlation is needed. In this work we describe a Danish family suffering from SYNS1 due to a novel *NOG* gene mutation (C230Y). We provide detailed clinical description of the family members presenting rare phenotype of the shoulders shared by affected individuals but no hearing loss, further adding to the phenotypic variability of the syndrome. With these findings we broaden the understanding of *NOG*-related-symphalangism spectrum disorder.

KEY WORDS

Facioaudiosymphalangism syndrome, NOG gene, noggin, proximal symphalangism, hyperopia, SYNS

1. INTRODUCTION

Multiple synostoses syndrome (SYNS; OMIM#186500) is a rare autosomal-dominant disorder characterized by a wide range of clinical manifestations. The symptoms include dysmorphic facies with a broad, hemicylindrical nose and a thin upper lip, ankylosis of the proximal interphalangeal joints often accompanied by multiple fusions in other joints, brachydactyly, conductive hearing loss caused by staples fixation and hypermetropia (1). Large variation of the phenotype is observed both between and within the diagnosed families (2). Three subtypes of the disorder can be distinguished (SYNS1-3) depending on the underlying genetic cause. Mutations in the gene encoding Noggin (*NOG*), a secreted bone morphogenic proteins (BMPs) signaling inhibitor, give rise to SYNS1 and a number of milder allelic conditions (2). These include Proximal Symphalangism, Teunissen-Cremers Syndrome and Brachydactyly type B. Due to overlapping clinical features of these disorders a collective term of *NOG*-related-symphalangism spectrum disorder (NOG-SSD) was created. SYNS2 and 3 are caused by haploinsufficiency of growth differentiation factor 5 (*GDF5*) and fibroblast growth factor 9 (*FGF9*) respectively (3, 4).

In this work a Danish family diagnosed with SYNS1 caused by a novel mutation in the *NOG* gene is described.

2. MATERIALS AND METHODS

After thorough medical examination, the patients were directed for mutation screening of the *NOG* gene. DNA was extracted from the peripheral blood of the patients indicated in the pedigree (figure 1A) using standard techniques. The coding region of *NOG* was PCR-amplified using GoTaq polymerase (Promega Corporation, Madison, USA). Primer sequences and cycling conditions are available upon request. Sequencing was carried out with the use of ABI BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA). Reactions were purified by BigDye XTerminator Purification Kit (AB) and subsequently sequenced with the use of ABI 3130 Genetic Analyser (AB). *In silico* evaluation of the genetic findings was performed with a set of prediction programs (Mutation taster, PolyPhen-2, SWIFT). Detected variants were evaluated in ExAC browser for their presence in publicly available exomes of over 60000 control individuals.

3. RESULTS

3.1 CLINICAL EVALUATION OF THE PATIENTS

The pedigree of the discussed cases is illustrated in figure 1A.

Patient 1, a 39-year-old male with nonconsanguineous Danish parents was referred to the genetics department with his newborn son (patient 2) because the family was under suspicion of arthrogryposis. The father was born at term with a weight of 4,65 kg (+2,5 SD) and the height of 56 cm (+2,5 SD). At present time he measures 197cm and presents with a

hemicylindrical nose with bulbous tip, short philtrum, slightly upslanting palpebral fissures and narrow shoulders (figure 2). He was suffering from recurrent pain in the knees, fifth fingers, toes and shoulders. Physical examination revealed reduction in joint mobility, especially in the shoulders and fifth proximal interphalangeal (PIP) joints. Lateral rotation of the shoulder was found reduced to 70 degrees (with normal range of 0-90^{o}). There was limited retraction of the scapula and the distance between the two margo medialis of scapula under maximal retraction was 10 cm. No scoliosis could be observed. There was a lack of a flexion crease at the fourth and fifth PIP bilaterally. Brachydactyly of the fifth fingers was observed, along with syndactyly between 2nd and 3rd finger and to a lesser extent between the 3rd+4th and 4th+5th fingers. He was unable to form a fist. X-ray of both hands revealed synostosis of the PIP joints of the 4th and 5th fingers in both hands (figure 2D). X-ray of the right shoulder showed normal osseous and articular conditions however a mild acromioclavicular joint osteoarthritis was detectable (figure 2E). The patient suffered from hypermetropia (+4.25/+7.5). Intelligence was normal. Screening for loss of hearing using a vibrating tuning fork was normal and tympanometry, auditory reflex test and an audiometry showed no signs of hearing loss. The patient was previously diagnosed with porphyria cutania tarda and also identified as a carrier of hemochromatosis.

Patient 2, a 5-month-old male presented with similar hand malformations as the father (figure 3C and 3D). Brachydactyly of the fifth fingers and similar pattern of syndactyly was observed. His birth weight was 3,55 kg (0 SD) and birth length was 51 cm (0 SD). Physical examination showed a 7,5 kg (0 SD) boy, with the length of 68 cm (0,5 SD) and the head circumference of 43 cm (-0.5 SD). At the age of fourteen months his weight was 9,9 kg (-1 SD), the length was 81 cm (0,5 SD) and his head circumference was 46,5 cm (-0,5 SD). Reduction in joint mobility was observed in the shoulders together with a lack of a flexion crease at the fourth and fifth PIP bilaterally. Lateral rotation of the shoulder was found reduced to 80 degrees. There was limited retraction of the scapula and the distance between the two margo medialis under maximal retraction was 8 cm. Flexion and abduction of the shoulder was reduced to 0-170 degrees (normal range 0-180^{o}).
X-ray of both hands at the age of 13 months showed cutaneous syndactyly between the 2nd and 3rd finger bilaterally, but no osseous abnormalities were shown (figure 3E). X-ray of the shoulders at the age of 13 months was normal. Since symphalangism was expected but not detectable at this stage, the child was further investigated using x-ray and a CAT scan of both hands (figure 3F) at the age of 16 months with 2D and 3D reconstructions. This showed no osseous adhesions, specifically no bone adhesions corresponding to the clinically observed lack of flexion crease at the 4th and 5th PIP joint bilaterally. However there was reduction of the joint space of the 5th PIP joint bilaterally, thus we suspect the child to have an element of fibrous or cartilaginous symphalangism in the 5th PIP joint bilaterally. The 5th middle phalanx was bilaterally short and plop in appearance, in comparison with the rest of the phalanges which appear normal, with no osseous pathology in the 4th finger. Postnatal hearing screening revealed no signs of hearing loss. This patient also suffered from hypermetropia (+10,5/+9,75). Tympanometry, auditory reflex test and a brainstem audio-evoked response test were normal.

Patient 3, a 36-year-old sister of patient 1 suffered from multiple joint-related symptoms. Recurrent pain in the knees, fifth fingers, toes and shoulders was accompanied by reduction in joint mobility prominent in the shoulders and 4th and 5th PIP. As a child she often suffered from airway infections. Physical examination showed a tall girl with a height of 178,5 cm (1,5 SD) and a weight of 101 kg (3,5 SD). The head circumference was 58,5cm. She had a hemicylindrical nose with a bulbous tip and slightly upslanting palpebral fissures. Short and broad feet with halux valgus were noticed. The same, familial hand phenotype was observed with lack of flexion crease at the 4th and 5th PIP bilaterally, characteristic syndactyly pattern and brachydactyly of the fifth fingers. Both shoulders were found to show reduced flexion 0-140 degrees (normal range 0-180), reduced abduction 0-110 degrees (normal range 0-180), reduced lateral rotation 0-70 degrees (0-90). We found a reduced mobility of the scapula and the distance between margo medialis under maximal retraction was 4 cm.

X-ray of the shoulders revealed an irregular glenoid cavity with central osseous defect of the cavity on both sides (figure 4E). The glenoid cavity looked flat especially on the right side. The head of the humerus was normally configured. No scoliosis was observed. Intelligence was normal. Screening for loss of hearing using a vibrating tuning fork was normal and tympanometry, auditory reflex test and an audiometry revealed no signs of hearing loss. She also suffered from hypermetropia (+6/+3,75).

Patient 4, a 5-year-old son of patient 3 displayed the same hand malformations as the mother (figure 5C and 5D). In addition to the reduced mobility of the shoulders and inability to form a fist, a marked reduction in pronation of the elbow was observed. The boy measured 123,3 cm (2,5 SD) with the weight of 22,5 kg (1,5 SD). Head circumference was 52,5 cm (1 SD). Cubitis valgus, narrow shoulders and thorax were noted. Both shoulders were found to show reduced flexion 0-170 degrees (normal range 0-180), reduced abduction 0-170 degrees (normal range 0-180), reduced lateral rotation 0-80 degrees (normal range 0-90). Reduced mobility of the scapula was found but with a normal retraction of the scapula. X-ray of the shoulders showed that the glenoid cavity was poorly defined and increased in width on both sides. At the age of 3 years the boy was diagnosed with severe hypermetropia (+11/+11). This defect progressed to (+12/+12) at the age of 5. Intelligence was normal. Tympanometry, auditory reflex test and an audiometry were normal.

We were not able to investigate the other members of this family with symphalangism as they were not interested in participating. None of the healthy family members suffered from hypermetropia.

3.2 GENETIC ANALYSIS

Mutation screening revealed a heterozygous mutation in the coding sequence of the *NOG* gene in all four investigated family members (figure 1B). The detected variant (c.689 G>A) was not reported previously in patients or control samples and resulted in the substitution of cysteine in position 230 by tyrosine (C230Y). The mutation affects an amino acid conserved in all known homologues of the gene and involved in the formation of a known disulfide bridge (5). It was predicted to be damaging to the protein function by all tested prediction programs with probability of >0,99.

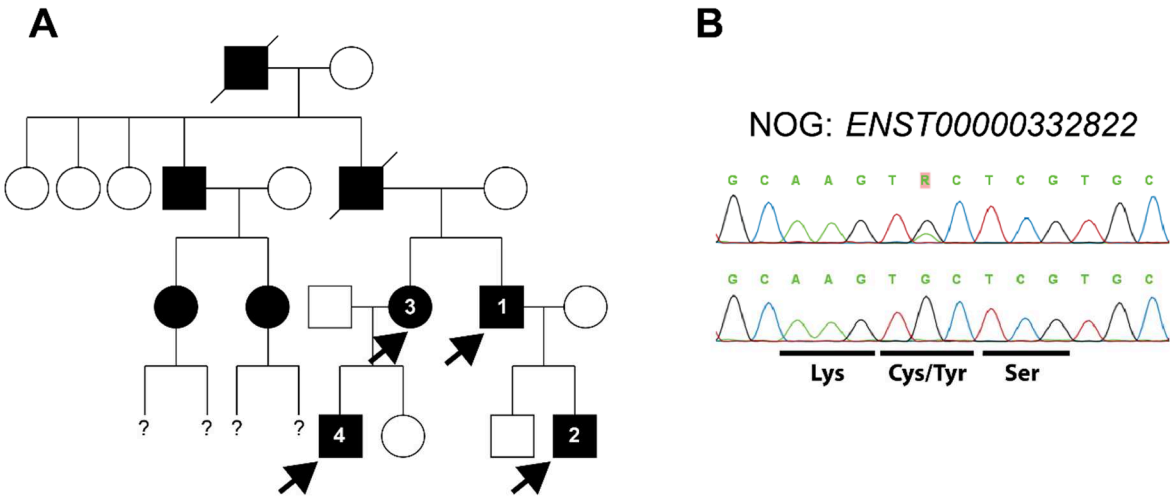

Figure 1: Pedigree of the family (A). Described individuals are indicated with arrows and numbers in respect to the text. (B) *NOG* mutation discovered in the family (top) and the sequence of an unrelated control individual (bottom).

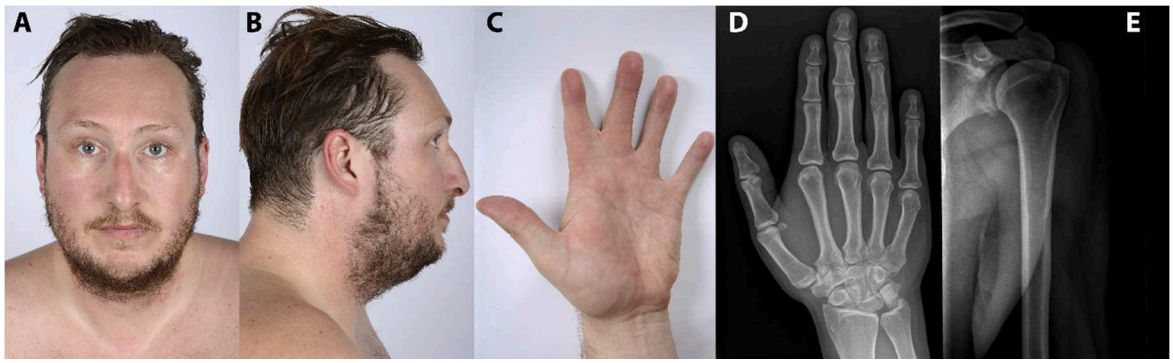

Figure 2: Clinical findings in patient 1. Facial features (A,B), familial hand malformation with lack of flexion crease at the 4th and 5th PIP joints (C), clear radiographical picture of symphalangism in the hand (D) and radiographic picture of the shoulder (E).

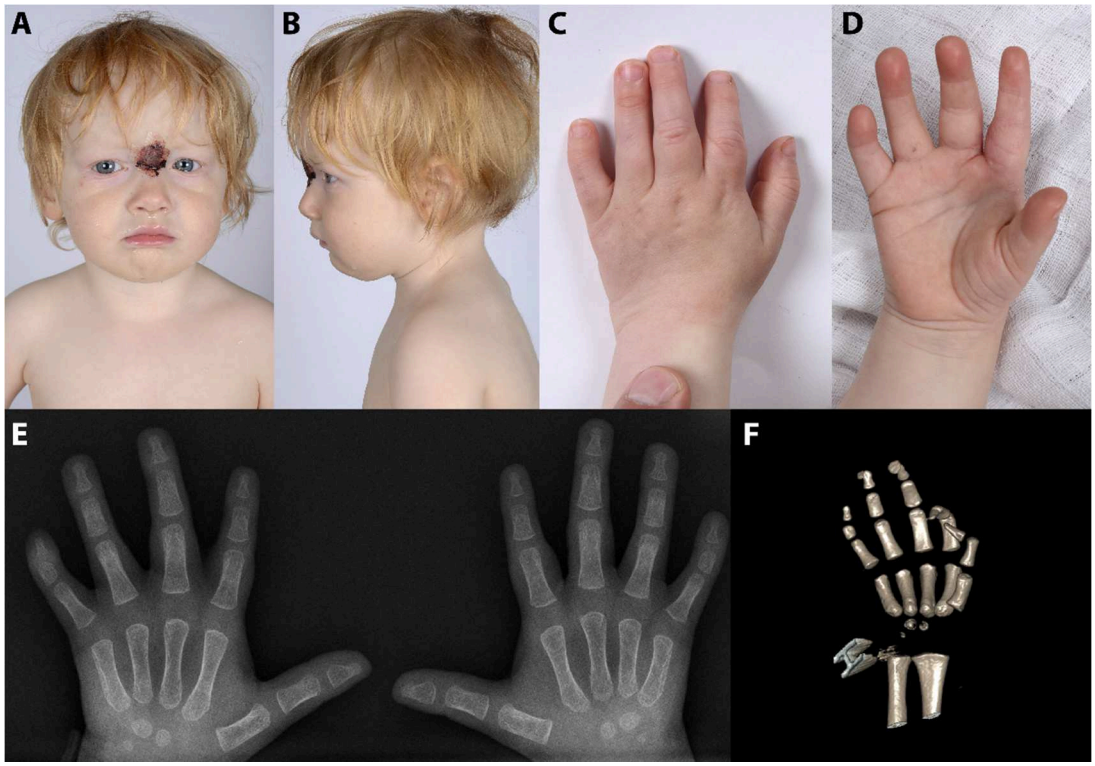

Figure 3: Clinical findings in patient 2. Facial features (A, B), hand malformations with apparent lack of flexion crease at the 4th and 5th PIP joint (C,D) radiographic picture of both hands (E) and 3D reconstruction of the hand obtained in CAT scan (F).

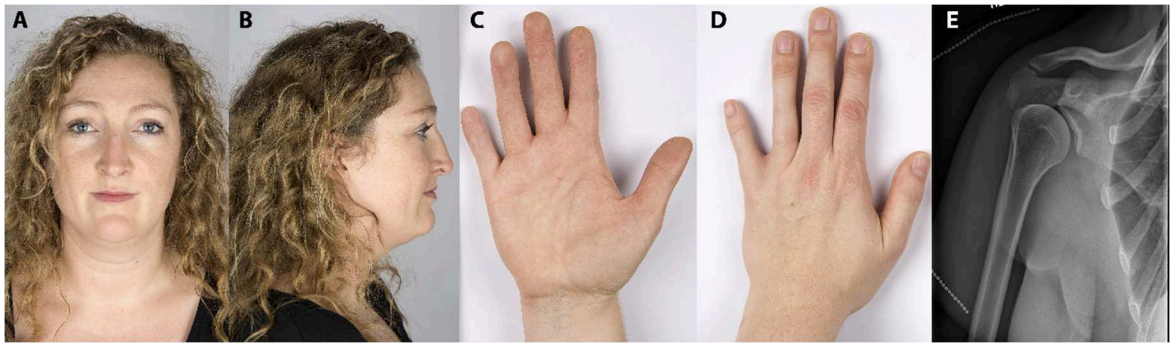

Figure 4: Clinical findings in patient 3. Facial features (A,B), hand malformations with visible lack of flexion crease at 4th and 5th PIP (C,D) and the x-ray of the shoulder (E) with deformed glenoid cavity.

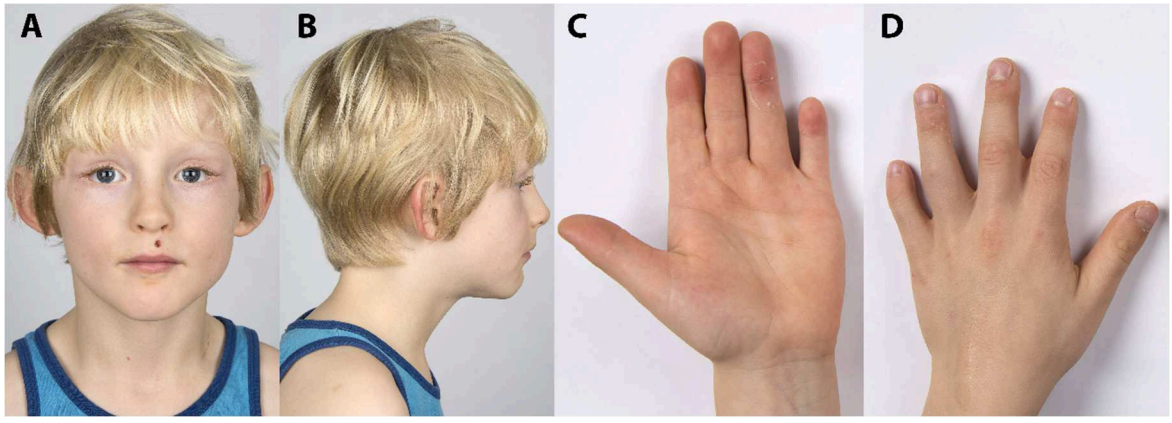

Figure 5: Clinical findings in patient 4. Facial features (A,B) and familial malformations of the hand (C,D).

4. DISCUSSION

The gene underlying proximal symphalangism was localized to chromosome 17q21-22 in 1995 by Polymeropoulus and colleagues (6). In 1998 Krakow *et al.* reported the association of multiple synostoses syndrome to the same region (7). One year later Gong and colleagues described first *NOG* mutations in patients diagnosed with multiple synostosis syndrome and proximal symphalangism (2). Since then more than 30 mutations in the *NOG* gene have been identified in patients suffering from a number of different conditions sharing proximal symphalangism as a common feature. The biological function of the protein encoded by this gene goes in line with the observed phenotypes. It has been shown that Noggin is a critical regulator of Bone Morphogenic Proteins (BMPs) signaling, a pathway implicated in a plethora of developmental processes including bone and joint formation (8, 9). Noggin is capable of binding and inhibiting BMPs, preventing their association to their cellular receptor and subsequent activation of a signaling cascade leading to the promotion of mesenchymal stem cell maturation and their differentiation into chondrocytes or osteoblasts (10-12). Given the major importance of these developmental events, tight, spatially and contextually accurate control mechanisms are essential. With mutations in *NOG* inflicting dominant negative effect on the functional, inhibitory, homodimeric form of Noggin a dysregulation of this pathway occurs, leading to observed pathologies (13). Multiple mutations in *GDF5* and *FGF9* have been shown to give rise to similar phenotypes but only *NOG* mutations were described to cause the full spectrum of SYNS features.

In this work we discuss a family diagnosed with multiple synostoses syndrome inherited in a clear autosomal-dominant manner. A novel *NOG* mutation has been found to underlie the phenotype of these patients leading to a substitution of cysteine residue at position 230 with tyrosine. Strong conservation of this amino acid among species, the nature of the amino acid substitution, the reported presence of mutations correlated with similar phenotypes in its close vicinity (Y222C, P223L, C232W) and the fact that the affected amino acid has been shown to form a disulfide bridge with cysteine at position 184 in crystallographic studies, allows us to confirm the mutation causality (2, 5, 14).

All described members of the family share common features of the disease. Hand malformations with clear symphalangism are observed in radiographic pictures of every affected individual except for the youngest family member. In this case, despite the lack of the flexion crease and inability to form a fist no ossified changes have been observed in x-rays nor CAT scans of the patient. This raises questions about the mechanisms underlying the formation of joint fusions in SYNS patients. The patient will be closely followed-up to further elucidate the progressive nature of this phenomenon. Deformations detected in the acromioclavicular joints, causing recurrent pain were more prominent in older patients reflecting the progressive nature of the disease. The fact that patients described in this study do not display conductive hearing loss typical for SYNS further illustrates the variability of phenotypes observed in patients bearing *NOG* mutations. Interestingly, no hearing impairment was also detected in a sporadic Japanese case, 3-years-old girl, diagnosed with

proximal symphalangism where *NOG* mutation in cysteine 184 was found (15). This amino acid is known to form a disulfide bond with cysteine 230 affected in the described family (5). The Japanese patient lacked any further features of the disease but it has been shown by Dixon *et al* that identical mutations in *NOG* can lead to different phenotypes depending on genetic background, suggesting the existence of epistatic modifiers of the gene (16). Together, these findings might also suggest some degree of genetic redundancy in the formation of the inner ear as fusion of the stapes to the temporal bone usually occurs in the patients bearing *NOG* mutations early in life (17, 18). It is important to remember that, with the progressive nature of SYNS a close monitoring of patients bearing *NOG* mutations is essential, as spine malformations, narrowing of the intervertebral space or hearing loss can occur during lifetime requiring a surgical intervention.

5. ACKNOWLEDGMENTS

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GENERAL DISCUSSION

The skeleton is a highly specialized organ providing the organism with a mechanical scaffold, shielding vital organs and serving as a reservoir of ions. Despite its static appearance, bone is in fact a metabolically active and dynamic tissue undergoing constant remodeling throughout life. This process requires a carefully controlled balance between bone formation and resorption in order to maintain the required strength and quality of the skeleton (1). Disruptions in this **dynamic equilibrium** often lead to bone diseases ranging from osteoporosis, in case of prevalent resorption, to sclerosing bone dysplasias on the other side of the spectrum. In the most recent update on the nosology of skeletal diseases more than 430 different osteochondrodysplasias were distinguished demonstrating the enormous complexity of observed phenotypes (2).

Over the years, major advances in the treatment of common bone phenotypes have been made through scientific efforts combining ***in vitro*** **studies**, such as appropriate cell cultures, with *in vivo* information obtained from **model organisms** (3, 4). However, as illustrated by several examples discussed in the first chapter of this thesis, many of the available therapeutics are based on the insights into the skeletal biology obtained through studies of diseases affecting human bone. In this way, **gene identification** has been placed at the forefront of the drug target discovery, leading to better understanding of the biology of bone and involved molecular pathways.

Gene identification strategies

In the past, the identification of causative mutations in rare diseases was mainly obtained by **positional cloning** efforts, starting from **linkage analysis** in large families. After associating the specific genomic region with the disease, mutation screening was performed to identify the specific mutation causative for the observed phenotype. Alternatively, **functional cloning** strategies, were employed to discover novel genes involved in pathways previously associated with bone based on sequence similarity (5).

In recent years, continuous developments in the field of **next generation sequencing** (NGS) allow great improvements in classical gene identification strategies. With sequencing costs plummeting down to below 500 USD per exome and little more than 1000 USD per genome, large scale, hypothesis-free investigations become available for larger study cohorts. **Exome sequencing** allows for parallel and deep sequencing of nearly 180000 protein coding exons accounting for approximately 1% of human genomic sequence, thus minimizing the costs per sample (6). However, despite these clear advantages, many challenges remain in the identification of causative variants with NGS techniques. Effective **variant filtering** strategies are necessary to properly differentiate between disease causing mutations and natural genomic diversity. The average human exome harbours up to 10000 variations altering protein sequence (7). As a consequence, even after excluding known, common polymorphisms and verifying segregation in available family members, one is often left with a large number of potentially pathogenic variants. Commonly used prioritization strategies employ ***in-silico*** **prediction programs**, estimating the potential effect of the identified variant on protein function based on sequence conservation and the nature of the

detected variation. To strengthen this approach, **endophenotypes** can be distinguished within the cohorts of patients suffering from the same disorder. In this way, patients displaying the same sets of symptoms can be investigated together in search of common mutations, or variation within the same genes.

Next to exome sequencing, a custom **targeted enrichment** strategy is commonly used as an enhancement of classical candidate study approach, allowing for cost-efficient inclusion of large gene panels in gene discovery. Currently, the sequencing using panels harboring genes with their known interaction partners, or even entire small pathways, became feasible with development of target enrichment kits, such as Nextera, HaloPlex, SeqCap EZ or MIPS technology. This approach is also commonly used for diagnostic purposes for a rapid mutation screening of multiple genes (8).

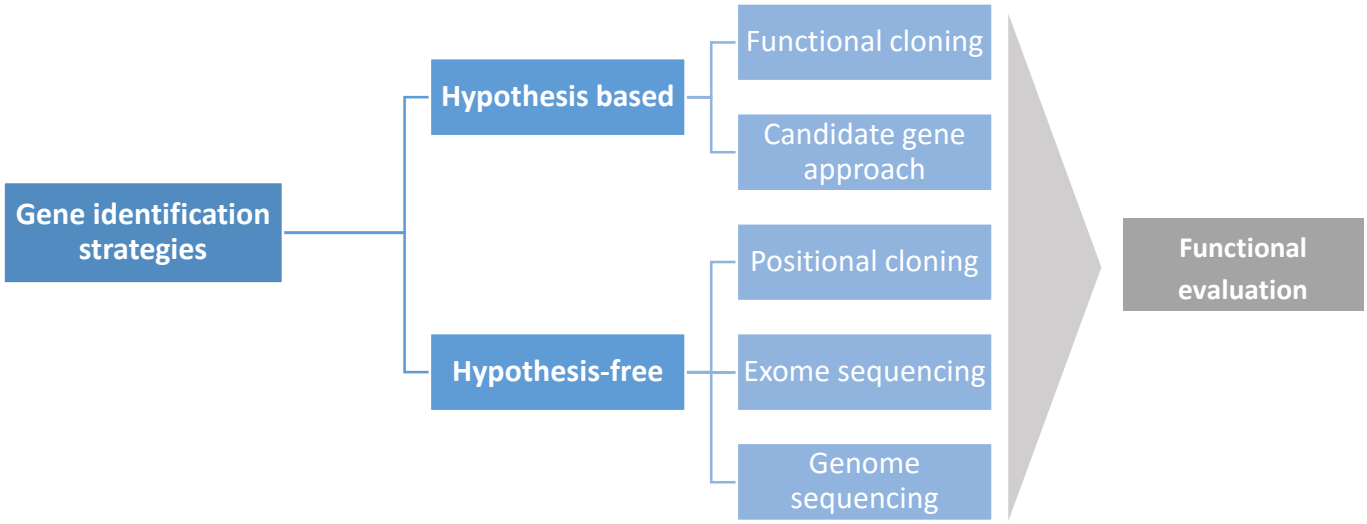

Figure 1: Schematic overview of the most commonly applied strategies for the gene identification in human disease.

Next to major developments in the detection of point mutations, NGS techniques can be used to strengthen currently available methods of detecting **structural variation** in the human genome. Copy number variations (CNVs) larger than 1kb are commonly omitted by classical sequencing techniques and can affect a significant portion of the human genome (9-11). Currently, CNVs are commonly detected by **SNP genotyping arrays** but recently, low-coverage whole genome sequencing has been presented as an effective alternative (12).

Recent developments in the analysis of whole genome sequencing data allowed to incorporate positional cloning efforts within the next generation sequencing pipelines. **Homozygosity mapping** technique is used to detect homozygous genomic regions inherited from both parents by genotyping short tandem repeats and SNPs, enhancing the gene identification for autosomal recessive disorders in consanguineous, isolated but also outbred populations (13-15).

Gene identification in craniotubular hyperostosis patients

Over the years, studies of the **craniotubular hyperostosis** patients cohort, described in the 4th chapter of this thesis, greatly contributed to our understanding of skeletal biology. Currently, this heterogeneous group of individuals gathers more than 60 isolated cases diagnosed with different forms of craniotubular hyperostosis without a known genetic cause. In the past, the causative genes for both sclerosteosis and Van Buchem disease have been localized to the region q12-21 on chromosome 17 (16, 17) with the help of larger families suffering from such conditions. Subsequent studies have shown that sclerosteosis is in fact caused by inactivating mutations in the *SOST* gene, encoding a secreted antagonist of the Wnt signaling pathway- sclerostin, while Van Buchem disease is caused by a 52kb deletion including a regulatory element modulating the expression of the same gene (18-23). These findings, together with the identification of *LRP5* mutations as causative for the autosomal dominant high bone mass phenotype and osteoporosis-pseudoglioma syndrome strongly established the role of **canonical Wnt signaling** in the regulation of skeletal health in humans and marked genes involved in this pathway as interesting targets in **candidate gene approach** screenings (24, 25).

To date, multiple point mutations in genes involved in canonical Wnt signaling have been found to cause different forms of sclerosing bone dysplasias as reviewed in the 1st and 2nd chapter of this thesis. Most notably however, a proteomic screen of interaction partners of sclerostin identified **LRP4** as a specific receptor and facilitator of the inhibitory action of this protein in canonical Wnt signaling. This finding has been confirmed by the identification of two point mutations in the *LRP4* gene to be disease causing in two, unrelated patients diagnosed with sclerosteosis (26). Further analysis of the *LRP4* gene in this thesis resulted in the identification of a novel point mutation in this gene in a Portuguese sclerosteosis patient described in detail in chapter 3. The candidate gene approach was also shown to be successful in our study of the Danish facioaudiosymphalangism syndrome family presented in chapter 5 of this thesis. In this study, the identification of a novel point mutation in the *NOG* gene provided new insights into the genotype-phenotype correlation in this condition.

All patients diagnosed with different forms of craniotubular hyperostosis included in our cohort undergo routine mutation screening of *LRP5*, *SOST* and *LRP4* genes, but despite these efforts no causative variants are found in a large proportion of these patients. As a consequence, we included new, **emerging candidate genes** in mutation screening efforts in this highly informative cohort. This strategy was also used in the mutation analysis of the novel family of the canonical Wnt signaling agonists- **R-spondins**, described in the 4th chapter of this thesis. At the beginning of this project, not much was known about this family of four, small, secreted proteins apart from their stimulatory effect on this pathway. We decided to screen our cohort of patients in search of rare, activating mutations that could possibly lead to observed phenotypes. Unfortunately, this approach was not successful in this study.

Moreover, in search for novel causative mutations in a hypothesis-free manner we perform whole exome sequencing on individuals from the population of craniotubular hyperostosis patients. However, distinguishing causative variants from polymorphisms is especially difficult in case of isolated, unrelated patients. To overcome this, aforementioned strategy of endophenotypic grouping can be employed. This approach provides great promise for our cohort of craniotubular hyperostosis patients but requires **detailed clinical information** about the investigated individuals.

Genetic determinants of complex traits

Studies of rare bone diseases can directly translate into the discovery of potent therapeutic targets for the treatment of common phenotypes affecting the skeletal system, as corroborated by multiple examples reviewed in the 1st chapter of this thesis. These findings extend the available knowledge on genetic determinants of bone parameters but fail to explain a large portion of natural variation observed in bone-related parameters in the general population. Currently, most of our knowledge on this matter comes from large-scale **genome-wide association studies (GWAS)**. These studies allow us to assess the genetic contribution of common variants to bone parameters across the whole genome in a hypothesis-free and high-throughput manner. Currently, the largest study available in relation to BMD and fracture risk is a 2012 meta-analysis study from the Genetic Factors of Osteoporosis (GEFOS) consortium (27). In total, over 60 genomic loci have been associated with BMD to date, highlighting the skeletal importance of several pathways including canonical Wnt signaling and the RANK-RANKL-OPG system (28). Among these, R-spondin 3 has been associated with BMD in a previous GWAS study (29). To further investigate this association, we decided to search for functional genetic variation in the R-spondin genes with high resolution. This is important, as variants detected by GWAS are rarely causative and functionally relevant, but rather remain in **linkage disequilibrium** (LD) with the causal variants. These variants are often located outside the gene highlighted by GWAS, as recently demonstrated in the case of the obesity-associated *FTO* locus where causal variants were detected in the downstream repressor sequence of *IRX3* and *IRX5* genes. These findings highlight the need for thorough verification of GWAS signals with genetic, *in vitro* and *in vivo* studies. The latter benefit from current developments in the field of **genome editing** allowing for drastic decrease in the cost and time needed to generate murine models with the use of the CRISP/Cas9 system (30, 31).

In case of R-spondin genes, we performed a **candidate gene association study** in stratified subpopulations of the healthy, young men from the Odense Androgen Study. These individuals were grouped based on their bone mineral density (BMD) t-scores, forming two equally sized groups representing the highest and lowest 10% of BMD scores in the population (as described in chapter 4). This approach allowed to test the effect of common variation in *RSPO* genes on BMD, a strategy previously successfully applied to discover a functionally relevant common variant in the *WNT16* gene (32). Unfortunately, in case of R-spondins no significant associations were detected. The OAS population has been

previously shown to possess at least 80% power to detect small genetic effects on BMD (~0,6%) with high accuracy (33). We speculate that the major importance of R-spondins in developmental events could possibly be the main illustration of proteins' involvement in skeletal health, therefore minimizing the effect seen in healthy individuals.

Interestingly, despite the large number of associated loci coming from GWAS, only ~6% of the heritable genetic variation in BMD can be explained by detected polymorphisms. Furthermore, recent efforts to account for the collective effect of all genetic variations in associated genes were insufficient and a large portion of the genetic variation in BMD remains unexplained (34). Several sources of this **missing heritability** have been proposed over the years and are likely to contribute to the observed phenomenon. First of all**, rare variants** displaying **larger effect sizes** are omitted by current GWAS studies, as current study designs only include common variants. Moreover, in order to obtain sufficient statistical power required to detect such variants, larger sample sizes are needed. In 2013, the first large scale, whole genome sequencing study has shown an association of a rare variant in *LGR4* gene, encoding the membrane receptor of R-spondins, with BMD and other parameters (35). More recently, another large-scale sequencing study was performed by GEFOS combining information from nearly 2900 genomes, ~3500 exomes and imputed data from the whole UK10K and 1000 Genomes projects to identify additional rare variants associated with BMD and fracture risk (36). Most notably, a non-coding variant localized downstream of engrailed homeobox 1 (EN1) has been shown to display significant association with several bone parameters, providing strong evidence that rare variants are likely contributors to the missing heritability. Moreover, an increasing line of evidence suggests that the **noncoding RNAs**, such as long noncoding RNAs (lncRNAs) and miRNAs hold a substantial part of regulatory and functional information encoded in the genome, which might be affected by genetic variation (37, 38). These relatively understudied genomic elements are increasingly characterized and catalogued in data repositories such as LNCipedia, which allowed to create customized microarrays to study the expression of (very often low abundant) lncRNAs (39). Deep sequencing of ribosome-protected RNA fragments recently questioned the noncoding character of many genomic regions (40, 41). In fact, some lncRNAs are found to be associated with translating ribosomes and produce peptides detectable by mass spectrometry (42, 43). It has also been shown that lncRNAs may be important metabolic modulators and potential drug targets (44, 45). Additionally, **CNVs** are likely contributing to the missing heritability phenomenon but a large degree of inconsistency is observed among the currently available studies, highlighting the need for novel study designs (46-48). Ultimately, **epigenetics** is increasingly recognized as a potent source of genetic variability that is currently understudied within the bone field. The term epigenetics refers to the heritable changes in the genome independent of the DNA sequence. Among other mechanisms, these changes include methylation, acetylation and phosphorylation of the histones. One example is the association of the acetylation status of the *SOST* promotor region with the gene expression (49). To add to this, several studies demonstrated the effect of DNA methylation on the expression of the known key modulators

of bone biology, including *SOST*, *OPG* and *RANKL* ultimately confirming the involvement of epigenetic mechanisms in the determination of bone mass (50, 51).

Altogether, the constant pursue of novel genetic factors implicated in skeletal biology yields many interesting candidates for the development of a new generation of drugs for the most common bone diseases, such as osteoporosis, presenting the biggest socioeconomic burden on our population.

Search for novel osteoporosis therapies

Currently available osteoporosis treatments focus mainly on preventing net bone loss by modulating the resorption rates. Based on efficacy, **bisphosphonates** are considered to be the first line of treatment with proven effect in reducing the risk of all nonvertebral fractures by 17%, hip fractures by 31% and vertebral fractures by 48% (52). The most commonly used bisphosphonates, alendronate, risendronate and zoledronic acid display high affinity to hydroxyapatite crystals, abundant components of the bone matrix. Bound to bone, they are taken up by the resorbing osteoclasts, directing the cells towards apoptotic death and therefore lowering bone resorption (53). Despite clear advantages and convenient administration (oral, quarterly or yearly intravenous injections), long-term safety of bisphosphonates can be an issue. Side effects can range from gastrointestinal events (54, 55) to renal dysfunction (56), atypical subtrochanteric and femoral fractures (57), and even osteonecrosis of the jaw (58).

As an alternative to bisphosphonates, **denosumab** - a fully humanized antibody against the receptor activator of nuclear factor-κB ligand (RANKL), is commonly used in osteoporosis treatment. RANKL is a known activator of osteoclast precursor cells that enhances their proliferation and differentiation towards mature bone-resorbing cells (59). Despite high efficacy at reducing fractures, with 68% reduction in vertebral fractures and 40% reduction in hip fractures after 36 months of treatment (59), several safety issues have been associated with this treatment. Most importantly, expression of RANKL on the cells of the immune system indicates the potential risk of altering the immune response of patients. It has been shown that patients undergoing denosumab treatment suffer from higher rates of serious infections when compared to a placebo group (60). Moreover, denosumab has been shown to significantly suppress the markers of bone turnover (61).

Several other strategies of lowering bone resorption remain in standard use in the clinical management of osteoporosis. **Strontium ranelate** was approved for this treatment in the EU, showing promising results in preventing vertebral fractures and general osteoporotic fractures, but no effect in reducing the risk of hip fractures (62). However, with the possibility of severe adverse effects, including multiorgan failure, this treatment is only recommended for severely osteoporotic patients unsuitable for other available therapies (63). Similarly, **selective estrogen receptor modulators** (SERMs) have been shown to effectively reduce the risk of vertebral fractures but their generalized, systemic effects raise safety concerns (64). Increased incidence of uterine polyps formation and nearly 50%

increase in the number of fatal strokes in women at high risk of cardiovascular complications remain important factors in inclusion criteria for this treatment (65, 66).

Altogether, the limitations of antiresorptive therapies, together with their inherent inability to reverse changes that already occurred, directed the scientific attention towards the development of alternative treatment strategies. In this regard, **anabolic treatments**, enhancing the formation of new bone, seem to be the most promising research avenue.

To date, only one anabolic osteoporosis therapy is available on the market. **Teriparatide**, the recombinant analog of human parathyroid hormone (PTH, aa 1-34), has been shown to successfully stimulate bone formation and reduce the risk of vertebral and nonvertebral osteoporotic fractures by 65% and 35%, respectively (67). The major safety concern accompanying the teriparatide treatment came from preclinical studies showing an increased incidence of osteosarcoma in rats subjected to prolonged PTH treatment (67). However, human studies did not confirm this effect with only three cases of osteosarcoma observed in over 16000 individuals undergoing PTH treatment over the last 15 years, a number that does not exceed osteosarcoma incidence in the general population (68-70). Despite that, the duration of the treatment has been limited to 24 months and the therapy is discouraged in patients at higher risk of osteosarcoma (such as Paget's disease patients). Together with the need for daily, subcutaneous injections, these facts indicate the urgency for the development of valid alternatives.

As discussed in chapter 1, several modulators of the canonical Wnt signaling pathway have been extensively studied as potential targets for such treatments. However, in the context of the research presented in this thesis, antibodies against **sclerostin** are the most relevant. Sclerostin, the potent secreted inhibitor of the canonical Wnt signaling pathway and bone formation, is expressed exclusively by late osteoblasts and osteocytes and is known to bind to the pathway co-receptor- LRP5 therefore preventing it from entering the pathways' activator complex (71, 72). Limited expression of the protein is likely to reduce the off-target effects of anti-sclerostin treatment. Neutralization of sclerostin by the humanized, monoclonal antibodies romosozumab and blosozumab, has been shown to markedly increase bone mineral density in recently concluded phase II clinical trial (73, 74), with an efficacy greater than bisphosphonates (alendronate) or teriparatide.

In light of these findings, LRP4, identified as a specific receptor and facilitator of the sclerostin action, shows great promise as drug target in future treatments of osteoporosis. In this work we provide insights into the genotype-phenotype correlation in patients bearing point mutations in the *LRP4* gene. We show that mutations responsible for impaired sclerostin binding localize exclusively to the central cavity of the third β–propeller domain of the protein, mimicking the phenotypes observed in patients bearing inactivating mutations in the *SOST* gene encoding sclerostin. These data indicate that developing a small molecule highly specific for this region of the protein could be a potent alternative for sclerostin neutralization. These findings are strongly supported by the recent generation of two antibodies neutralizing the sclerostin facilitator function of LRP4 (75). These antibodies

have been shown to specifically block sclerostin-dependent inhibition of the canonical Wnt signaling without disturbing the binding between LRP4 and agrin, critical for the functioning of neuro-muscular junctions. Marked increase in bone mass and related parameters has been observed in rats subjected to the treatment using these antibodies, clearly demonstrating the major importance of *LRP4* in the regulation of bone mass. To further study the biological consequences of LRP4 deficiency we decided to generate a **murine knock-in model** of the novel sclerosteosis mutation identified in this thesis-p.R1170Q. Preliminary, unpublished data suggest that our LRP4 mutant KI mice show marked increase in the thickness of femoral cortexes (figure 2) and cranial bones when compared to wild-type littermates, resembling the phenotype observed in human subjects. At the moment, extensive breeding is performed in order to obtain sufficient number of animals for statistically sound data analysis. We strongly believe that further investigation of this model will provide us with novel insights into the mode of action of LRP4.

Figure 2: μCT femoral cross sections from WT mouse (A) and p.R1170Q homozygous knock-in littermate (B).

In conclusion, with the work presented in this thesis we broaden the understanding of several osteochondrodysplasias. Some of the novel insights provide the ground for future translational studies aiming at widening the spectrum of available treatments for common bone phenotypes.

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ABBREVIATIONS

AD	Autosomal dominant
ADO	Autosomal Dominant Osteopetrosis
AP	Alkaline phosphatase
AR	Autosomal recessive
ARO	Autosomal Recessive Osteopetrosis
BMC	Bone mineral content
BMD	Bone mineral density
BMP	Bone morphogenic protein
CaCN	Calcineurin
CAT	Computerized axial tomography
CIAP	Calf intestinal alkaline phosphatase
CKO	Conditional knock-out
CLCN7	Chloride channel 7
CLS	Cenani-Lenz syndrome
CNV	Copy number variation
CRD	Cysteine rich domain
CTX	Carboxy-terminal collagen crosslinks
DKK	Dickkopf
DMEM	Dulbecco's Modified Eagle Medium
Dvl	Dishevelled
ECD	Extracellular domain
EDTA	Ethylenediaminetetrraacetic acid
EGF	Epidermal growth factor
Fz	Frizzled
HBM	High Bone Mass
IARO	Intermediate Autosomal Recessive Osteopetrosis

Krm	Kremen
LDL	Low-density lipoprotein
Lef	Lymphoid-enhancer binding factor
LGR	Leucine-rich repeat-containing G protein coupled receptor
LRP	LDL receptor related protein
GFP	Green fluorescent protein
Gpc	Glypican
GPI	Glycosylphosphatodylinositol
GWAS	Genome-wide association study
MIPS	Molecular inversion probes
MM	Multiple myeloma
NOG	Noggin
OA	Osteoarthritis
OAS	Odense Androgen Study
OB	Osteoblast
OC	Osteoclast
OPG	Osteoprotegerin
OPPG	Osteoporosis-pseudoglioma Syndrome
OVX	Ovariectomized
P1NP	Type 1 procollagen
PCP	Planar cell polarity
PDB	Pagets Disease of Bone
PG	Proteoglycan
PIP	Proximal interphalangeal joint
PKC	Protein kinase C
PTH	Parathyroid hormone

RANK	Receptor activator of nuclear factor κ B
RANKL	Receptor activator of nuclear factor κ B ligand
RSPO	R-spondin
SAMP6	Senescence accelerated mouse P6
SCID	Severe combined immunodeficiency
SCL	Sclerosteosis
SD	Standard deviation
SERM	Selective estrogen receptor modulators
sFRP	Secreted frizzled related protein
SGBS	Simpson-Golabi-Behmel syndrome
SNP	Single nucleotide polymorphism
SYNS	Multiple synostoses syndrome
TG	Transgene
VBD	Van Buchem Disease
Wif	Wnt inhibitory factor
Wnt	Wingless-type MMTV integration site family member
WT	Wild type

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Educational activities

- Master thesis co-supervision: Raphaël De Ridder, "Genetic and functional evaluation of the role of RIN3 in the pathogenesis of Paget's disease of bone.", University of Antwerp, promoter Wim Van Hul, Academic Year 2014-2015.
- Master thesis co-supervision: Ingrid Alexandra Szilagyi, "Genetic and functional study of RSPO 1, 2 and 3 in bone formation", Vrije Universiteit Brussel, Interuniversity Program of Molecular Biology (IPMB), promoter Wim Van Hul, Academic Year 2013-2014.
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Publications

Bayat, A *, **Fijalkowski, I** *, Andersen, T, Abdulmunem, S, van den Ende, J, Van Hul, W. (2016) *Further delineation of Facioaudiosymphalangism Syndrome: Description of a Family with a Novel NOG Mutation and Without Hearing Loss.* American Journal of Medical Genetics- Accepted for publication.
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Fijalkowski, I, Geets, E, Steenackers, E, Van Hoof, V, Ramos, F J, Mortier, G, Fortuna, A M, Van Hul, W and Boudin, E. (2016), *A Novel Domain-Specific Mutation in a Sclerosteosis Patient Suggests a Role of LRP4 as an Anchor For Sclerostin in Human Bone.* J Bone Miner Res. doi: 10.1002/jbmr.2782

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Scientific awards

- The Young Investigator Award of the International Skeletal Dysplasia Society for the presentation: "LRP4 mutations in sclerosteosis and Cenani-Lenz syndrome impair sclerostin action via different mechanisms.", August, 1st, 2015
- The Young Investigator Travel Grant of the American Society of Bone and Mineral Research for the abstract: “Identification of the Third LRP4 Mutation in a Patient Diagnosed with Sclerosteosis.”, September, 12th, 2014

Oral presentations

- **Igor Fijalkowski.** "Genetic and functional study of LRP4 and R-spondins in bone formation", European Calcified Tissue Society PhD training course, Ljubljana, Slovenia, 2011
- **Igor Fijalkowski,** Eveline Boudin, Ana Maria Fortuna, Geert Mortier, Feliciano J. Ramos, Peter Itin, Wim Van Hul**.** "LRP4 mutations in sclerosteosis and Cenani-Lenz syndrome impair sclerostin action via different mechanisms", International Skeletal Dysplasia Society, Istanbul, Turkey, 2015

Poster presentations

- **Igor Fijalkowski**, Eveline Boudin, Ellen Geets, Ana M. Fortuna, Geert Mortier, Feliciano Ramos, Peter Itin, Wim Van Hul. "Genotype-phenotype correlation of the mutations in the LRP4 gene causing Cenani-Lenz syndrome and sclerosteosis- investigating the disease mechanisms." Belgian Society of Human Genetics, Leuven, Belgium, 2016
- **Igor Fijalkowski,** Eveline Boudin, Torben L. Nielsen, Marianne Andersen, Kim Brixen, Wim Van Hul**.** "*Search and functional evaluation of rare variants in RSPO3 gene."*, European Calcified Tissue Society, Rotterdam, The Netherlands, 2015
- **Igor Fijalkowski**, Eveline Boudin, Torben L. Nielsen, Marianne Andersen, Kim Brixen, Wim Van Hul**.** "*Search and functional evaluation of rare variants in RSPO3 gene.*", Belgian Society of Human Genetics, Charleroi, Belgium, 2015
- **Igor Fijalkowski**, Eveline Boudin, Joao Silva, Wim Van Hul. "Identification of the third LRP4 mutation in a patient diagnosed with sclerosteosis", American Society for Bone and Mineral Research, Houston, USA, 2014
- **Igor Fijalkowski**, Vere Borra, Eveline Boudin, Wim Van Hul. "Expression analysis of mesenchymal KS483 cells during differentiation towards osteoblasts", European Calcified Tissue Society, Lisbon, Portugal, 2013
- Eveline Boudin, Elke Piters, **Igor Fijalkowski**, Gino Stevenheydens, Wim Van Hul. "Mutations in sFRP1 and sFRP4 are not a common cause of craniotubular hyperostoses". Belgian Society of Human Genetics, Liege, Belgium, 2012

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