Sclerostin

A Key Regulator of Bone Metabolism

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A Key Regulator of Bone Metabolism

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door

A.H.J.M. van Lierop

Geboren te ‘s Gravenhage 1981

Waar ik het tomeloos duister afzoek

Door tijd en donkere materie heen

Ontluikt, vanuit de verste uithoek

Een kleine ster, lichtjaren van z'n buur alleen

Voor miljarden jaren al bestaan

Slechts niet eerder door mens ontwaard

Nu schenk ik hem jouw naam

En zet hem daarmee op de kaart

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Contents

Preface

Preface

Bone is a living tissue. Throughout life the skeleton constantly maintains its structural integrity by replacing old or damaged bone with newly laid down bone by a process called remodelling. This process of bone remodelling is accomplished by a tight collaboration between bone resorbing osteoclasts and bone forming osteoblasts, working together as a basic multicellular unit (BMU) (Figure 1). Bone remodelling commences by attraction of osteoclast precursors to bone sites, where they further differentiate into mature multinucleated osteoclasts, which start to resorb bone, leaving behind a resorption cavity. Osteoblasts are subsequently attracted to this resorption pit by signals derived from the osteoclasts and stimulatory factors embedded in the bone matrix, released by resorption (1). Over a period of 3 to 4 months the resorption cavity is refilled with new layers of bone matrix, layed down by the osteoblasts, which subsequently mineralizes. In healthy adults, the amount of bone formed equals that initially resorbed. The activity of osteoclasts and osteoblasts are tightly regulated, by each other, but also by a third type of bone cell, the osteocyte (2,3). Osteocytes are terminally differentiated osteoblasts embedded in the bone. During the process of bone formation, about 10-20% of the osteoblasts are buried in the bone matrix by the advancing osteoblasts. These cells differentiate into osteocytes, developing long dendritic processes through which they keep contact with the osteoblasts on the bone surface. This way osteocytes can be found regularly scattered throughout the bone, making up for more the 90% of all bone cells, and forming a dense network with their dendritic pseudopods, through which they can communicate with each other and cells on the bone surface (4).

For many years osteocytes were thought to be involved in the regulation of bone remodelling, but due to their embedment in bone, these cells were difficult to study, and their exact function remained an enigma. However, recent studies illustrated that osteocytes are indeed the main orchestrators of bone remodelling. Osteocytes produce RANKL, a major stimulatory signal for osteoclast differentiation and activity, by which they can regulate bone resorption (6,7). Similarly osteocytes can control the rate of bone formation by synthesizing sclerostin, a key inhibitory signal for osteoblast activity and lifespan (8).

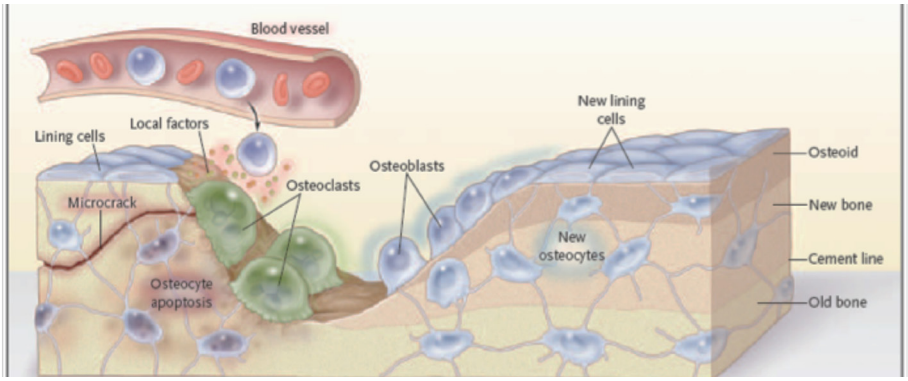

Figure 1. A schematic overview of the bone remodelling cycle. Lining cells and osteocytes release local factors that attract cells from blood and marrow into the remodeling compartment in which osteoclastogenesis occurs. Osteoclasts resorb matrix, then successive teams of osteoblasts deposit new lamellar bone. Osteoblasts that are trapped in the matrix become osteocytes; others die or form new, flattened osteoblast lining cells. Reproduced with permission from the New England Journal of Medicine (5), Copyright Massachusetts Medical Society

Disturbances in the balance between bone resorption and bone formation form the pathophysiological basis of many bone diseases. The most prevalent bone disease is osteoporosis, in which an increased bone resorption is not sufficiently compensated by bone formation, leading to gradual bone loss and deterioration of its microstructure leading to increased bone fragility (9). Conversely, there are several, less prevalent, bone disorders in which there is an imbalance in favour of bone formation, leading to increased accumulation of bone and thick bones. These sclerosing bone disorders can be caused either by impaired production or activity of osteoclasts (osteopetroses), or by increased production of osteoblasts (hyperostoses or osteoscleroses) (10). Sclerostin was discovered a decade ago by studies of two such bone sclerosing dysplasias namely, sclerosteosis and van Buchem disease (11-14). Both these disorders are the result of impaired sclerostin synthesis, leading to an imbalance of bone remodelling in favour of bone formation.

The aim of this Thesis is the investigation of the role of sclerostin in bone metabolism in humans. In Chapter 1 we review current knowledge of sclerostin, and the sclerostin-deficient disorders sclerosteosis and van Buchem disease. In Chapter 2, we describe the characteristics and performance of the assay we used to measure sclerostin levels

in blood. In Chapters 3 and 4 we report studies of patients and disease carriers of sclerosteosis and van Buchem disease, respectively. In Chapter 5 we present the results of treatment of van Buchem disease with glucocorticoids. In the subsequent Chapters we report studies of the regulation of sclerostin synthesis by systemic factors, such as glucocorticoids (Chapter 6) and PTH (Chapter 7) and the role of sclerostin in type 2 diabetes mellitus (Chapter 8), Paget's disease of bone and prostate cancer metastatic to the skeleton (Chapter 9).

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Chapter 1

The role of sclerostin in the pathophysiology of sclerosing bone dysplasias

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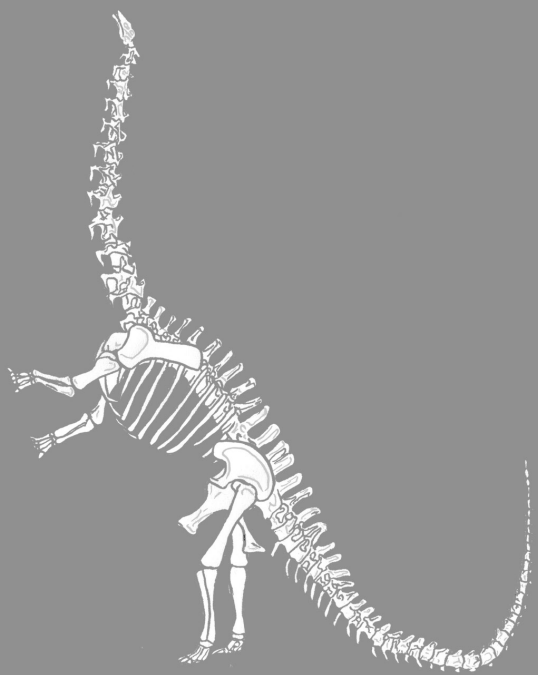


Clinic Rev Bone Miner Metab. 2012;10:108-116

Introduction

Osteocytes, the most abundant cells in bone, are terminally differentiated osteoblasts burried in the bone matrix which are key regulators of bone remodelling and have also important functions in the regulation of mineral metabolism [1]. Osteocytes act on both osteoclasts and osteoblasts. They synthesize receptor activator of nuclear factor kappa-B ligand (RANKL) [2,3], which is essential for osteoclast proliferation, differentiation and survival, and are the main source of sclerostin which inhibits bone formation by the osteoblasts [4]. Whereas animal models were pivotal for the discovery of RANKL and its role in bone resorption [5], it is human studies of two rare bone sclerosing dysplasias, sclerosteosis [6,7] and van Buchem disease (VBD) [8,9] that have led to the discovery of sclerostin. Both these disorders are caused by deficient synthesis of sclerostin, resulting in unrestrained bone formation and progressive generalized hyperostosis.

We review here current knowledge of the mechanism of action and the regulation of synthesis of sclerostin, and of its role in the pathophysiology of sclerosteosis and VBD.

Sclerostin synthesis

Osteocytes synthesize sclerostin in the late stages of their differentiation, after maturation and after the start of mineralization of the surrounding bone matrix [10] (figure 1). Newly synthesized sclerostin is then transported to the bone surface through the dendritic network of osteocytes, where it inhibits the activity of osteoblasts and stimulates their apoptosis [4,11]. Recent evidence, suggests that sclerostin has also an autocrine function [12]. Furthermore, sclerostin upregulates RANKL synthesis thereby stimulating osteoclastogenesis [13]. Although osteocytes are the predominant source of sclerostin, other cell types embedded in mineralized matrices, such as chondrocytes [11] and cementocytes [14], have also been found to produce sclerostin

Sclerostin is the product of the SOST gene, a relatively small gene comprising two exons, situated on chromosome 17q12-q21. The SOST gene is highly conserved among vertebrates, with the amino acid sequence of murine sclerostin being 88%

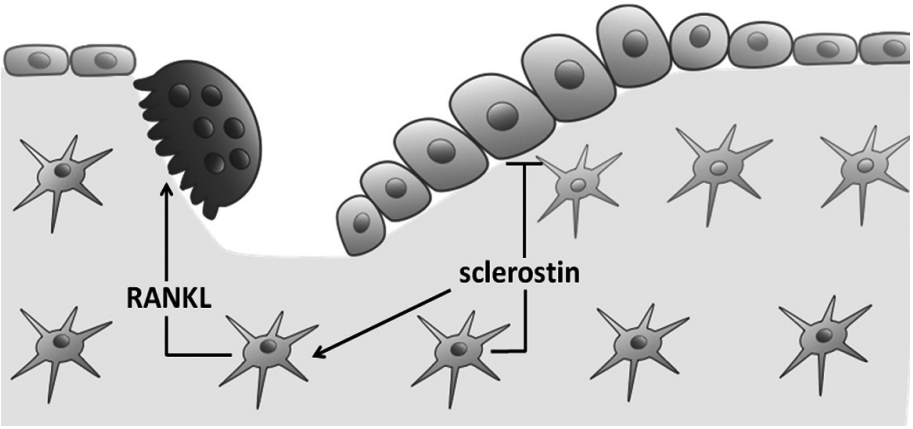


Figure 1. Role of sclerostin in bone remodeling. Sclerostin is synthesized by matured osteocytes at the initiation of mineralization of new bone. Sclerostin act on the osteoblasts on the bone surface, by inhibiting their bone forming activity and life-span. Sclerostin can also indirectly stimulate bone resorption by up regulating RANKL synthesis by osteocytes in an autocrine manner.

homologous with the human protein [6]. Moreover, SOST knock-out mice develop a high-bone-mass phenotype similar to that of sclerosteosis patients [15], whereas mice overexpressing SOST become osteopenic [11]. In addition to bone, cartilage and cementum, transcripts of SOST have also been found in kidney, liver, and heart [6,7], but no sclerostin expression could be detected in any of these tissues in human [16]. In keeping with this finding, patients with sclerostin deficiency have normal renal and liver function and no specific cardiac abnormalities [17-19].

Sclerostin antagonizes the canonical Wnt signaling pathway

Sclerostin decreases bone formation by antagonizing the canonical Wnt signaling pathway in osteoblasts thereby inhibiting the proliferation, differentiation and survival of these cells [20-22]. Secreted Wnt ligands bind to a co-receptor complex of the low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) receptor and a trans membrane frizzled receptor. Upon binding, intracellular β-catenin is prevented from degradation and accumulates in the cytoplasm. β-catenin is translocated to

the nucleus and triggers the transcription of target genes through the interaction with TCF/LEF-1 transcription factors [23] (figure 2A). Sclerostin binds to the first propeller domain of the $LRP_5/6$ receptor [21], thereby disabling the formation of the co-receptor complex between $LRP_5/6$ and the frizzled receptor, and inhibiting the Wnt pathway high up in the signalling cascade (figure2B). Whereas, the exact mechanism by which sclerostin interacts with the $LRP_5/6$ receptor remains to be established, it is thought that it requires an, as yet to be identified, co-factor to inhibit the Wnt pathway similar to another Wnt antagonist, DKK1, which needs Kremen to inhibit the pathway [24]. LRP_4 was recently proposed to be a mediator of sclerostin's inhibitory function on bone formation and mutations in LRP_4 were identified in patients with a phenotype closely resembling that of sclerosteosis [25]

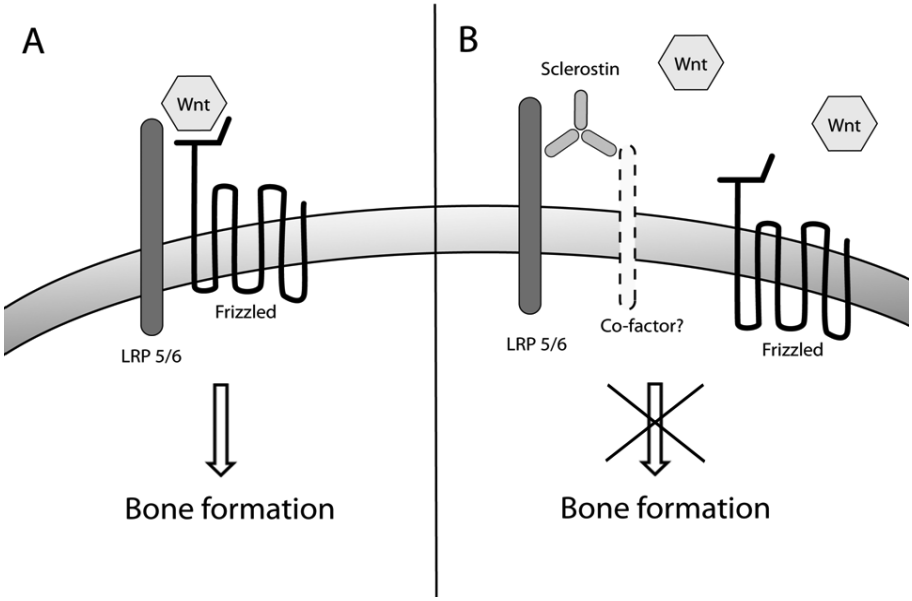


Figure 2. Mechanism of inhibition of canonical Wnt signalling by sclerostin
Sclerostin is an antagonist of the canonical Wnt signalling pathway. A: the Wnt signalling pathway is initiated by the binding of Wnt ligands to a co-receptor complex of LRP5/6 and the frizzled receptor. Wnt signaling is essential for osteoblast proliferation, differentiation and survival, and is thus the main stimulatory pathway of bone formation. B: sclerostin antagonizes the Wnt signalling pathway by inhibiting the formation of the LRP5/6-frizzled co-receptor complex, through binding to the LRP5/6 receptor, possibly facilitated by a (unknown) co-factor.

Sclerosing bone disorders associated with sclerostin deficiency

The significance of the role of sclerostin as a negative regulator of bone formation is highlighted by the characteristic phenotypes of patients with sclerosteosis and VBD, two bone sclerosing dysplasias belonging to the group of craniotubular hyperostosis [26] caused by genetically determined sclerostin deficiency, and characterized by very high bone mass.

Sclerosteosis (OMIM 269500) was described in 1958 by Trushwell as 'osteopetrosis with syndactyly' [27] and less than 100 cases have since been reported in the literature. Sclerosteosis is an autosomal recessive disorder caused by mutations in the SOST gene. Six different mutations have been reported so far resulting in either impaired synthesis of sclerostin [6,7,28], or synthesis of a non-functioning protein [29]. Although isolated cases of sclerosteosis have been reported in different parts of the world, the majority of patients are members of the Afrikaner community of South Africa, descendants of Dutch immigrants who settled in this country in the 17th century. Although the homozygous state is rare, the carrier rate of the SOST mutation in the Afrikaner population has been estimated to be as high as 1 in 140 individuals [30]. Van Buchem disease (OMIM 239100), first described by professor van Buchem and his colleagues in 1955 as 'hyperostosis corticalis generalisata familiaris' [31], is a sclerosing bone dysplasia also inherited as an autosomal recessive trait. In this disorder the SOST gene is intact, but patients lack a regulatory element essential for the postnatal transcription of SOST in bone [32] due to a 52 kb deletion 35 kb downstream of the SOST gene [8,9]. About 30 cases of VBD have been so far described, the vast majority being inhabitants of a small village in north Holland. This village used to be an island off the coast until extensive land reclamation connected it to the mainland in the 1940s. Two siblings with VBD have been reported by German investigators, but the origin of the patients was not mentioned in the paper [33] so possible Dutch ancestry cannot be excluded.

The clinical features of sclerosteosis have been extensively described [17,34,35]. The most prominent of these features are due to overgrowth of the bones of the skull. Mandibular overgrowth, and elongation of the forehead [17,34] result in facial distortion which becomes evident before the onset of puberty [17,34] (figure 3).

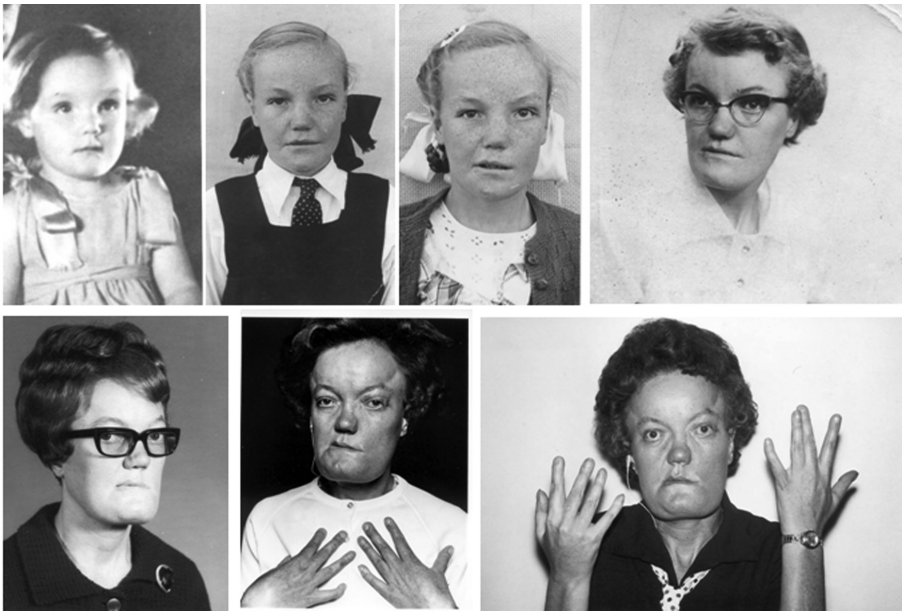


Figure 3. Chronological portraits of a patient with sclerosteosis from the age of 3 years onward. She was born with syndactyly at both hands and developed facial palsy, deafness, facial distortion, and maxillary overgrowth during childhood. By the age of 30, she had developed proptosis and elevated intracranial pressure due to overgrowth of the calvaria. Craniectomy was performed, but she died nevertheless because of elevated intracranial pressure at the age of 54 years (description of this case was previously published by Epstein et al.[36]). Reproduced with permission of Calcified Tissue International.

Excessive bone formation in skull bones eventually give rise to serious complications, the most common being cranial nerve entrapment syndromes due to obliteration of neural foramina (figure 4), with the facial nerve being the most frequently affected. This is often the first complication of the disease, occurring generally before the fifth year of life, although facial palsy may also be observed at birth [34,35]. Unilateral or bilateral facial palsy eventually develops in almost all patients [17,34,35,37]. Hearing loss is the second most frequently encountered complication of sclerosteosis. It usually starts in early childhood as pure conductive deafness, due to fixation of the ossicles in the inner ear, with a sensorineural component often developing later in life as a result of narrowing of the round and oval windows, or of impingement of the acoustic nerve in the internal acoustic canal [17,34,35,37]. Although rare, symptoms associated with entrapment of other cranial nerves, such as loss of vision or sense of smell have also

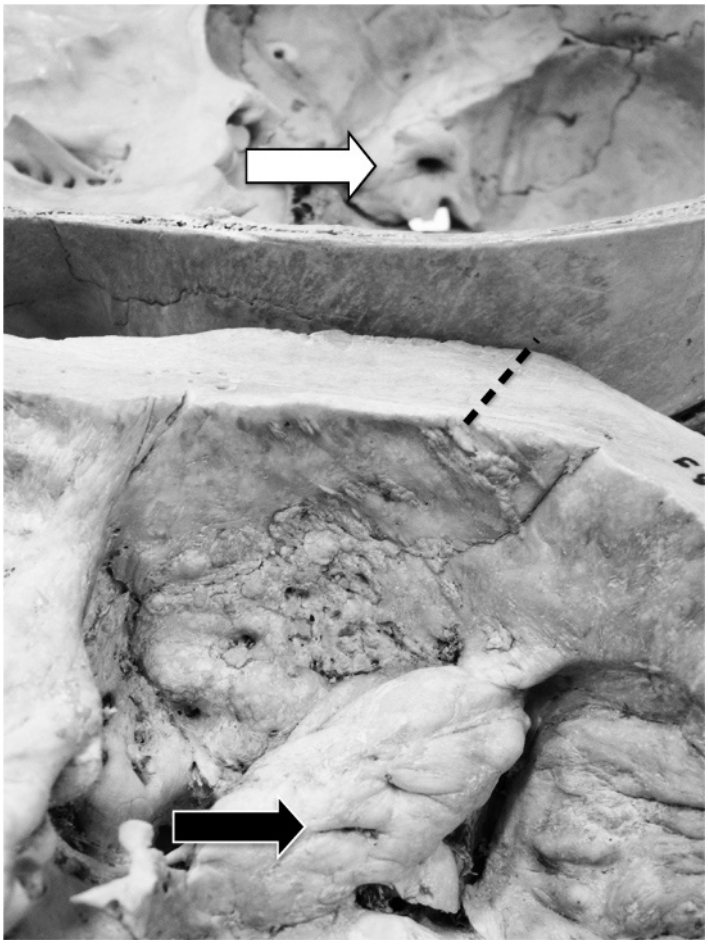


Figure 4. Skull of a sclerosteosis patient (lower panel) compared to that of a normal subject (upper panel). Severely narrowed internal acoustic meatus in the patient (black arrow) compared to that of the normal subject (white arrow). The greatly thickened calvarium of the patient can also be noted (dotted line).

been reported [34,37]. However, the most severe, and life-threatening complication of sclerosteosis is increased intracranial pressure [17,35]. This develops in the majority of patients as a result of a decreased intracranial volume due to considerable thickening of the calvaria and skull base. In the past this has been a common cause of sudden death of patients with sclerosteosis [17] due to medullary compression. Although the course of sclerosteosis is evidently progressive during childhood and adolescence, the disease appears to stabilize after the third decade [34,35,38].

The phenotype of patients with VBD is highly similar to that of sclerosteosis except for two distinctive features of sclerosteosis, which have so far never been reported in patients with VBD, namely syndactyly and tall stature [39] (Table 1). There can be, however, a great variation in the severity of disease manifestations among patients with VBD [39]; van Lierop et al in preparation]. While the phenotype of severe cases with VBD is highly similar to that of patients with sclerosteosis, other patients with VBD have mild abnormalities and only few lifelong complications. Facial distortion is a consistent feature of VBD [19] but less prominent than in sclerosteosis [39]. In the majority of patients facial palsy also develops in the first years of life [18,40], and had been also present at birth in some patients. The presence and severity of hearing loss varies greatly among VBD patients, being profound in some, but absent or mild in others, and can be purely conductive, purely sensorineural, or mixed [18,41]. Increased intracranial pressure is a rare complication of VBD [42]. As mentioned above, patients with VBD do not have syndactyly, possibly because the deleted SOST enhancer element does not regulate embryonic SOST transcription[32]. It is noteworthy that apart from the characteristic skeletal changes, the general health of patients with sclerosteosis and VBD is otherwise very good [17,19]. Remarkably the excessive bone formed in sclerosteosis and VBD is of very good quality, with patients sustaining no fractures even after severe trauma [17,41].

Tabel 1. Characteristics of Sclerosteosis and van Buchem disease

	Sclerosteosis	van Buchem disease
Genetic defect	Mutation in SOST gene	52 kb deletion downstream of SOST
Pattern of inheritance	Autosomal recessive	Autosomal recessive
Stature	Tall	Normal
Facial distortion	Severe	Moderate-severe
Syndactyly	Common	Absent
Facial palsy	Common	Common
Hearing loss	Common	Common
Increased intracranial pressure	Common	Rare

Pathophysiologically, van Buchem had already suggested some fifty years ago that the disease may be caused by excessive bone formation, rather than decreased bone resorption, as is the case in osteopetrosis [19]. Excessive bone formation has indeed been histologically demonstrated in bone biopsies obtained from patients with sclerosteosis [35,37] and biochemically by measurements of markers of bone turnover in patients with VBD [19,43] and sclerosteosis [35-37]. Consistent with the identified genetic defect, no sclerostin was detected immunohistochemically in osteocytes of patients with either sclerosteosis [4] or VBD [14]. Particularly interesting is the finding of a normal pattern of bone markers during growth in these patients, increasing during childhood and adolescence, but declining after cessation of the growth spurt to levels around the upper limit of the adult reference range [35,42] (figure 5). No abnormalities have been reported in serum calcium, phosphate and parathyroid hormone (PTH) concentrations [18,36,37] in either disease. There were no abnormalities in the pituitary hormonal axis when tested in a small number of patients with sclerosteosis [36].

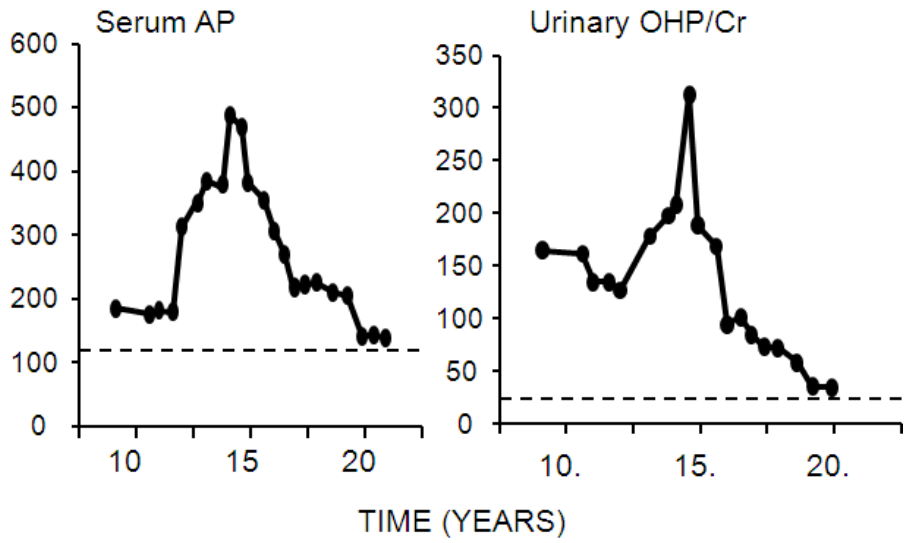


Figure 5. Sequential measurement of serum alkaline phosphate activity (AP) in U/l, and urinary hydroxyproline to creatinine ratio (OHP/Cr) in µmol/mmol in a patient with van Buchem disease over a 10-year period. Interrupted lines indicate the upper limit of the normal range.

Skeletal radiographs show generalized hyperdensity and increased endosteal thickening of the tubular bones, similar in patients with sclerosteosis and VBD [19,41,44]. These changes are reflected in measurements of bone mineral density (BMD) which is greatly increased at the hip and the spine with z-scores sometimes exceeding +10. On CT-scan obliteration of neural foramina, which can be decreased to less than 1 mm, is a common finding and the jugular canal was narrowed in a few cases [37,45]. In both sclerosing dysplasias radiographic changes usually become evident at the end of the first decade and progress up to the third decade of life when they appear to slow, at least in patients with sclerosteosis [34], Van Hoenacker and colleagues showed, however, progressive radiographic changes in the metacarpals of patients with VBD with ageing [41].

Treatment of sclerosteosis and VBD

There is to date no specific therapy available for the management of patients with sclerosteosis and VBD which remains so far largely symptomatic. Decompressive surgery may be needed to free entrapped nerves, and hearing aids can help to improve hearing. In a patient with a severe case of VBD the insertion of a ventriculo-peritoneal drain led to a reduction in intracranial pressure and improvement of symptoms [42] (figure 6). In sclerosteosis, placement of a ventriculoperitoneal or lumboperitoneal shunt do not give satisfactory results, in contrast to the improvement following anterior and/or posterior craniotomy [46]. We recently reported that glucocorticoids which decrease bone formation by reducing the number and function of osteoblasts may be a useful adjunct in the management of patients with severe disease [42].

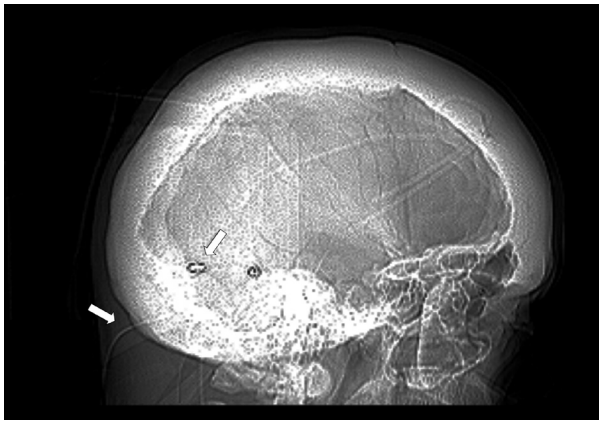
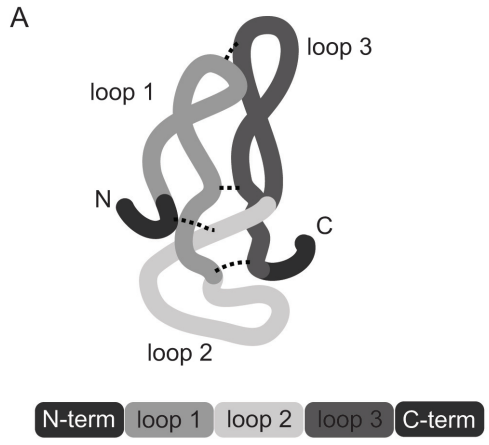


Figure 6. CT scan of the skull of a patients with VBD, demonstrating severe thickening of the calvaria and presence of ventricular liquor drains (arrows) for the management of increased intracranial pressure.

Measurement of circulating sclerostin

Assays for the measurement of sclerostin in serum of humans have recently become available. Using an assay developed by MSD (Gaithersburg, Maryland, USA) we could not detect (<1 pg/ml) any sclerostin in the serum of 19 patients with sclerosteosis. In contrast, sclerostin was detectable in serum of 77 healthy men and women tested. Serum samples from 7 patients with sclerosteosis were also measured with another, frequently used commercially available assay (Biomedica, Vienna, Austria). In 4 of the 7 patients no sclerostin was detected, while the protein was detectable in the 3 other with values overlapping with reported low normal values [47-49]. We have further validated the MSD assay by epitope mapping, and we tested the reactivity of sclerostin fragments of different lengths in both the MSD and Biomedica assay. These fragments consisted of the 3 loops without the N- and C-terminus, the 1st and 2nd loop without the N- and C-terminus, and the 3rd loop alone (figure 7A). High concentrations of the fragments (10ng/ml) were not detected by the MSD assay, while all were detected by the Biomedica assay (figure 7B).

Figure 7. Measurement of fragments of sclerostin molecule using 2 different commercially available sclerostin assays.



A: Simplified representation of the sclerostin molecule. Sclerostin is a glycoprotein consisting of 190 residues, which form a 3 loop like structure, with a cystine knot (dotted line) at the base [50,51]. The 3rd loop is considered to be the binding site to LRP5, while the 2nd loop was found to be the binding site of an inhibiting monoclonal antibody against sclerostin [51], implying an important function for this loop. The long C- and N-termini are also of importance for sclerostin action, for a sclerostin variant lacking only the C- and N- terminus had reduced activity [50].
B: detection of sclerostin fragments of different length by the MSD and Biomedica assay.

These findings illustrate the difficulties in the interpretation of data from different assays which do not detect the same sclerostin forms, as also demonstrated by McNulty et al [52]. These authors showed that sclerostin values measured with the Biomedica assay correlated poorly with those measured with another commercial assay (TECO, Sissach, Switzerland). Until more is known about the secreted, circulating as well as bioactive forms of sclerostin, caution is called for in the clinical interpretation of results of circulating sclerostin.

Factors affecting the synthesis of sclerostin

In animal studies, mechanical loading of bone has been shown to stimulate the expression of SOST and sclerostin in osteocytes while unloading had the opposite effect [53,54]. Consistent with these findings, patients immobilized after a stroke, and thus deprived from mechanical load, were shown to have increased serum sclerostin levels, which were negatively correlated with the bone formation marker serum bone specific alkaline phosphatase [55]. However, a more recent study of immobilized patients due to spinal cord injury reported opposite results and it was suggested that mechanical unloading may have different, time-dependent, effects on sclerostin production [56].

Hormonal factors have also been shown to modulate the synthesis of sclerostin both in animals and humans. In animals, continuous [57] or intermittent [58] administration of PTH downregulates SOST expression and sclerostin synthesis, and serum sclerostin levels are decreased in patients with primary hyperparathyroidism, increasing after successful parathyreoidectomy [59]. In addition, treatment of postmenopausal women with teriparatide for 14 days were associated with significant decreases in serum and bone marrow levels of sclerostin [60]. These results suggest that the anabolic effect of PTH on bone is exerted, at least in part, by a decrease in the production of sclerostin. Recent studies have also indicated that estrogens may also influence sclerostin synthesis. Postmenopausal women have been reported to have higher circulating sclerostin levels than premenopausal women, these were negatively associated with bone formation markers [48], and decreased by estrogen replacement therapy [47]. Estrogen deficiency may, therefore, adversely affect the

skeleton not only by increasing bone resorption but also by directly reducing bone formation by increasing sclerostin synthesis, thus, further deteriorating the imbalance between bone resorption and bone formation which is the pathophysiological basis of postmenopausal osteoporosis. In contrast, testosterone replacement therapy was reported to increase serum sclerostin levels in hypogonadal men [47].

Alterations in sclerostin synthesis may also be involved in the pathogenesis of bone lesions in inflammatory bone and joint disorders. The pro-inflammatory cytokines tumor necrosis factor (TNF) and TNF-related weak inducer of apoptosis (TWEAK) were reported to upregulate SOST expression in vitro and ex vivo [61]. Patients with ankylosing spondylitis have also been reported to have lower serum sclerostin levels compared to healthy subjects and to patients with rheumatoid arthritis and sclerostin levels were found to be associated with the formation of new syndesmophytes [62]. Sclerostin expression was also greatly reduced in joints of patients with ankylosing spondylitis compared to those of patients with rheumatoid arthritis or osteoarthritis. These results, suggest a specific alteration of the function of osteocytes in patients with ankylosing spondylitis. Therapeutic agents known to have deleterious effects on bone by increasing bone loss and fracture risk, such as glucocorticoids and thiazolidinediones, were also reported to upregulate SOST expression in osteocytes in vitro [63,64]

Sclerostin as a new therapeutic target for the management of osteoporosis

The restricted expression of sclerostin to the skeleton and its extracellular activity, made this protein an attractive candidate for the development of a new bone forming therapy for the management of osteoporosis. This approach was further supported by the gene-dose effect suggested by findings in heterozygous carriers of sclerosteosis who demonstrate decreased serum sclerostin levels associated with increased levels of P1NP [35] and high normal or increased BMD [65] without any clinical symptoms, signs or complications of sclerosteosis. Over the past few years, neutralizing antibodies against sclerostin (Scl-ab) were developed and tested in several animal models. When given to overiectomized rats, an antibody significantly increased the

rate of bone formation at all skeletal envelopes, increased bone mass and improved bone strength [66]. The Scl-ab, importantly, also clearly increased bone formation in the periosteum, a site hardly affected by current treatments of osteoporosis, while it appeared to decrease bone resorption. When given to nonhuman primates Scl-ab dose-dependently increased bone formation which was associated with increases in bone mass and strength [67]. In a Phase 1 clinical trial a single subcutaneous or intravenous injection of Scl-ab administered to healthy men and women led to a rapid and dose-dependent increase in serum P1NP, and to an increase in BMD at the spine and hip within 3 months of administration [68]. Consistent with findings from animal studies, the administration of the Scl-ab also decreased serum CTX, a marker of bone resorption. Phase 2 clinical studies of the efficacy and tolerability of SCL-ab are currently underway.

Treatment with Scl-ab has also been shown to prevent inflammation- induced bone loss in mice with chronic colitis and glucocorticoid-induced bone loss in mice on dexamethasone treatment [69,70]. The effect of Scl-ab on fracture healing was also recently studied in animal models and was shown to increase bone mass and strength at the site of fracture, to improve callus formation and maturation, and to reduce the incidence of non-union [71]. A Phase 2 clinical study of the effect of Scl-ab on fracture healing is in last stages of completion.

Conclusion

The story of sclerostin is a true example of how genetic studies of rare diseases can lead to ground breaking new insights into molecular pathways, and how the understanding of the pathophysiology of a disorder may lead to the development of new therapies for another. Sclerostin has been shown to play a key role in bone metabolism, and sclerostin inhibition by neutralizing antibodies might prove to be a very successful therapy in restoring bone mass in low bone mass disorders such as osteoporosis. Unraveling the role of sclerostin in the pathophysiology of the disabling and sometimes life-threatening disorders sclerosteosis and VBD has, unfortunately, not yet led to the development of a satisfactory therapy for their management. Effective therapies to control the unrestrained bone formation associated with these disorders are clearly needed

Acknowledgements

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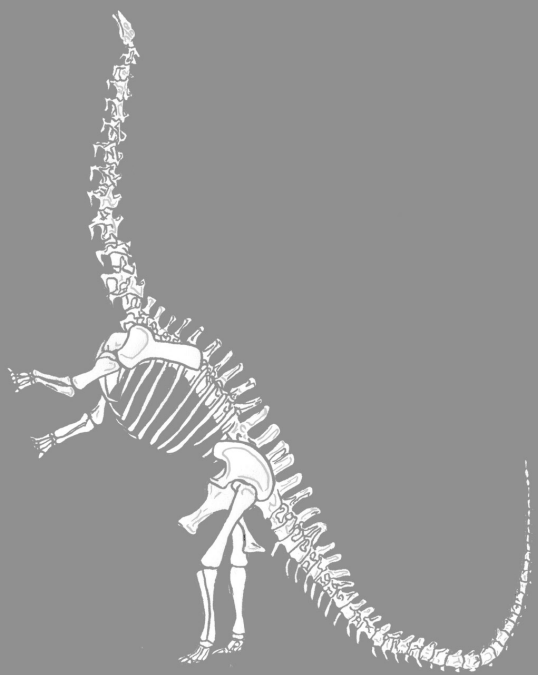
Chapter 2

Measurement of circulating sclerostin

A.H. van Lierop
S.E. Papapoulos



Introduction

Sclerostin is a glycoprotein secreted by osteocytes (1) (Figure 1), which acts locally on cell membrane receptors of osteoblasts (2) and in an autocrine fashion on other osteocytes (3). Sclerostin is released in the circulation and is detected in serum and plasma (4). There is a strong relationship between circulating and bone marrow plasma levels of sclerostin consistent with the findings that osteocytes are the major source of sclerostin production and, therefore, the sclerostin present in peripheral blood [5]. There are currently 3 commercially available assays for sclerostin. Two of these, manufactured by Biomedica and TECOmedical gruppe (TECO), are conventional enzyme-linked immunoabsorbent assays (ELISA), based on chemiluminescence. The third assay, manufactured by Meso Scale Discovery (MSD) is an ELISA based on electrochemiluminescence. In all studies reported in this thesis sclerostin was measured by the MSD sclerostin assay (MSD® 96-well MULTI-ARRAY® Human Sclerostin Assay). Prior to these studies we examined the performance of this assay according to international guidelines (6;7). In this Chapter we describe the results of these experiments.

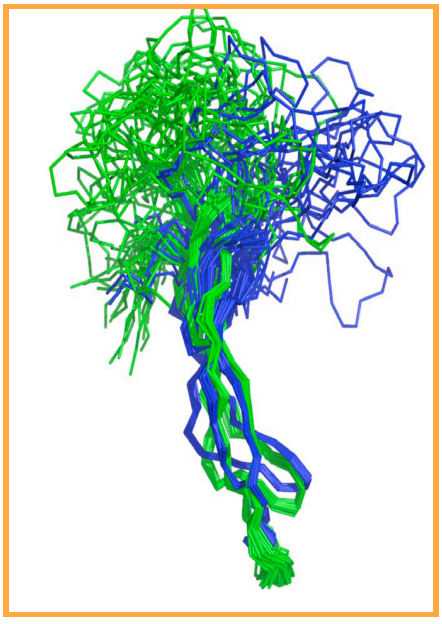


Figure 1. Structure of sclerostin
(green = human sclerostin; blue = mouse sclerostin)

Description of the MSD sclerostin assay

In the MSD assay, 96 well plates are coated with a purified goat polyclonal antibody raised against the full murine sclerostin molecule as capture antibody; a polyclonal goat anti-human sclerostin is used as detection antibody. The detection antibodies are tagged with electrochemiluminescent labels, which emit light when electrochemically stimulated. The detection process is initiated at electrodes located in the bottom of the microplates. Background signals are minimal because the stimulation mechanism (electricity) is decoupled from the signal light [http://www.mesoscale.com/CatalogSystemWeb/WebRoot/technology/ecl/walkthrough.htm. accessed January 2012 (8)]. The sclerostin standard for the assay was produced in an NS0-derived myeloma cell line, and the purity was checked by SDS–PAGE gel with silver stain.

Characterization of the MSD sclerostin assay

Bioavailability of circulating form(s) of sclerostin is currently unknown and the specificity of commercial assays for different domains of the molecule has not been reported. To assess the specificity of the MSD assay for the sclerostin molecule, we performed a series of experiments.

First, to validate the sclerostin standard used in this assay, we compared it to that of recombinant sclerostin prepared by the University of Würzburg, Germany, kindly provided by Professor Thomas Mueller within the TALOS research consortium. This recombinant sclerostin was produced in an E.Coli strain, and the protein concentration was accurately determined by spectrophotometry. Serial dilutions (10 to 1000 pg/ml) of both these sclerostin preparations were measured. Values of the two peptides were highly concordant at every concentration. The mean ratio of the concentrations was 1.02 (range 0.8-1.3) (Figure 2).

Second, to characterize the specificity of the antibodies used in the assay, we performed epitope mapping of the polyclonal detection and capture antibodies according to Pepscan's Epitope Mapping Technology [kindly performed by Jaap Willem Back and Peter Timmerman of Pepscan Therapeutics (Lelystad, the Netherlands) within the TALOS research consortium]. Pepscan's Epitope Mapping Technology uses microarrays of overlapping peptides, covering the complete sequence of a given protein. Antibody binding studies subsequently identify the peptides representing the

protein interaction of interest. (http://www.pepscantherapeutics.com/technology/epitope-mapping). The epitope mapping revealed two binding sites of the capture antibody for human sclerostin, one on the N-terminus and one on the distal end of the C-terminus. For the detection antibody there were three apparent epitopes; at the N-terminus, at the 3rd loop, and at the C-terminus of the protein (Figure 3). We validated these results by assessing the reactivity of sclerostin fragments of different sizes (provided by Jaap Willem Back of Pepscan Therapeutics). We tested three different fragments comprising the three loops without the N- and C-terminus; the first and third loop without C- and N-terminus; and the second loop alone (Figure 4). All fragments were added at high concentrations (10 ng/ml). As expected from the epitope mapping, all fragments were undetectable, suggesting that the assay detects the whole sclerostin molecule.

Third, to assess the specificity of the assay for sclerostin we tested serum samples of 3 patients with sclerosteosis. These patients do not synthesize sclerostin, due to a genetic defect in the gene encoding sclerostin. As expected, no sclerostin could be detected in the serum of all 3 patients. In contrast, sclerostin was detected in serum of all 77 healthy individuals tested. These results demonstrate that the MSD assay is highly specific for the sclerostin protein and, hence, suitable for clinical studies.

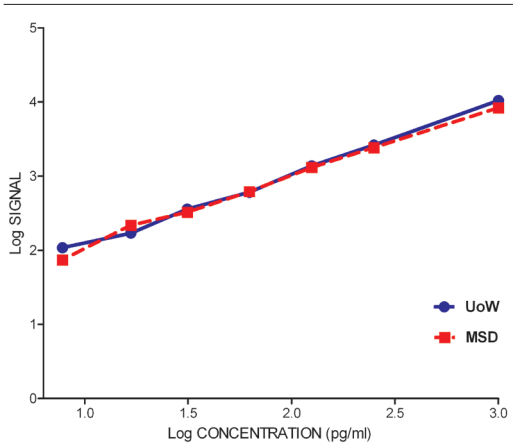


Figure 2. Comparison of serial dilutions of the Meso Scale Discovery (MSD) sclerostin standard with recombinant sclerostin prepared by the University of Wurzburg (UoW). When measured with the MSD sclerostin assay, the output signal of both sclerostin preparations were highly concordant at all concentrations.

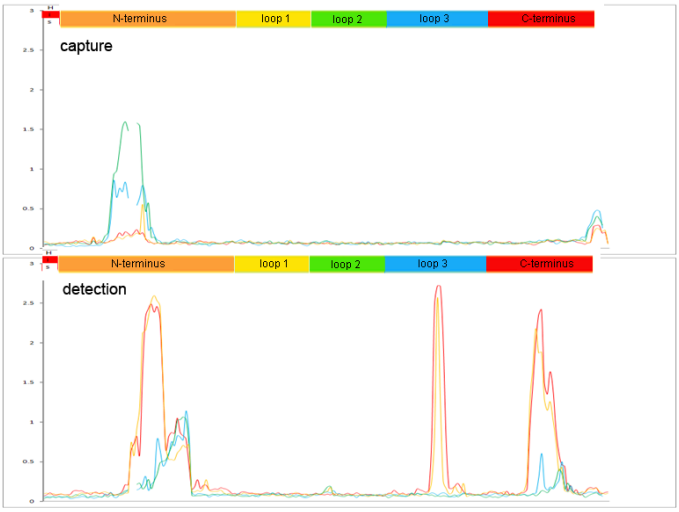


Figure 3. Epitope mapping of the MSD capture antibody (upper panel) and detection antibody (lower panel). Spikes in the base line represent binding affinity of the antibodies for the sclerostin molecule.

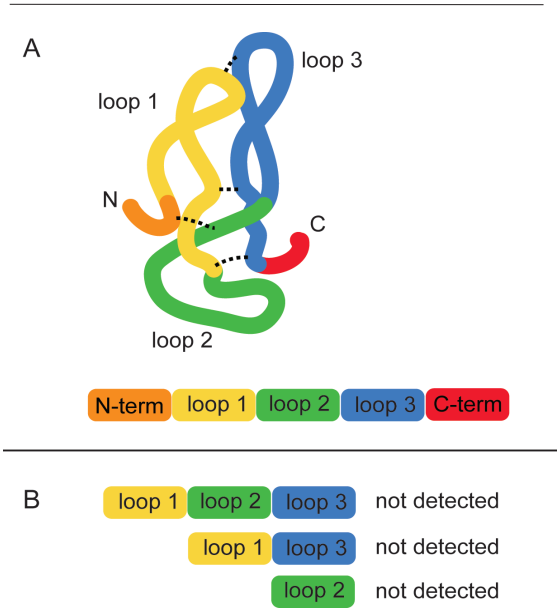


Figure 4. Measurement of fragments of the sclerostin molecule using the MSD sclerostin assay.
A: Simplified representation of the sclerostin molecule.
B: measurement of sclerostin fragments of different sizes with the MSD sclerostin assay. All fragments were undectable.

Performance of the MSD sclerostin assay

The performance of the MSD sclerostin assay was assessed according to international guidelines (6;7) and results are summarized in Table 1. The relationship between measured sclerostin values of serially diluted samples and the outcome signal was linear (r=0.998). The lower limit of detection, determined by the mean +2 standard deviations of the output signal of 10 blank samples, was 1 pg/ml.

Table 1. Performance of the MSD sclerostin assay

Test	Results
Lower limit of detection	1 pg/ml
Detection range	1-10,000 pg/ml
Inter assay precision (CV)[a]	10 %
Intra assay precision (CV)	6 %
Recovery[b]	82 -92 %

CV= coefficient of variation ([standard deviation/mean]*100)

[a] excluding samples at the end of the spectrum of the detection range (10 and 10.000 pg/ml)

[b] recovery of exogenously added sclerostin to serum sample

Within-run precision and between-run precision were determined at 5 different concentrations spanning the detection range (10, 50, 100, 1000, and 10,000 pg/ml). The within-run precision was less than 6% at all concentrations. Between-run precision was below 10% at 50, 100 and 1000 pg/ml, but somewhat higher at 10 pg/ml (13%) and 10,000 pg/ml (15%). In 3 serum samples measured over 10 runs the between-run precision was less than 7% (Table 2). The recovery of exogenously added sclerostin (65, 125 and 250 pg/ml) to serum samples was 82%-93%.

Table 2. Inter assay precision assessed in 3 serum samples measured in 10 different runs

Sample	Mean concentration (pg/ml)	Standard deviation (pg/ml)	Coefficient of variation (%)
1	20.98	1.54	7.4
2	37.70	2.32	6.2
3	106.34	7.89	7.4

To assess the stability of the sclerostin protein in serum subjected to repeated freezing and thawing, we made aliquots of freshly drawn serum samples. One aliquot was thawed and frozen for 7 subsequent runs, and compared to a fresh aliquot during each run. For each run, measured sclerostin values in the previously thawed aliquots were highly concordant with those of the single thawed aliquots (86-98%); there was no trend for increasing or decreasing sclerostin values in subsequent runs.
The stability of measured sclerostin in serum kept at room temperature (20ºC) was determined by storing aliquots of 3 different serum samples at room temperature for 8, 24 or 48 hours. Aliquots were then frozen and were measured in the same assay and compared to aliquots immediately frozen after blood collection. Results are shown in Table 3. After 8 hours at room temperature, serum sclerostin levels decreased by 60% in one sample whereas these were 94% and 120% of levels measured in the immediately frozen sample, in the other two samples. After 24 hours, measured sclerostin levels decreased by 33%-68% of the original levels in all 3 samples and decreased further slightly after 48 hours at room temperature (23%-65%). For clinical studies it is, therefore, essential to store serum in the deep freeze soon after its separation from blood, a procedure that was followed in all studies reported in this thesis.

Table 3. Stability of sclerostin in serum kept at room temperature (20ºC)

	sample	1	2	3
	frozen	20,38	12,89	25,32
Period at 20ºC	8h	12,22 (60%)	15,51(120%)	23,75 (94%)
	24h	6,77 (33%)	6,96 (54%)	17,13 (68%)
	48h	4,60 (23%)	5,48 (43%)	16,47 (65%)

The effects of different sample media, serum or EDTA plasma, on measured sclerostin values was determined by measuring sclerostin levels in simultaneously drawn serum and EDTA plasma samples from 26 subjects. Sclerostin levels were higher in plasma by a factor of 3.6 ± 1.0 compared to those measured in serum, but levels in serum and plasma were highly correlated (r=0.91, p=0.001) (Figure 5). Similar findings have been previously reported for heparinized plasma and were attributed to the formation of complexes of sclerostin with other proteins in serum. (4). It should be

mentioned, however, that the sclerostin molecule has a binding site for heparin (9).

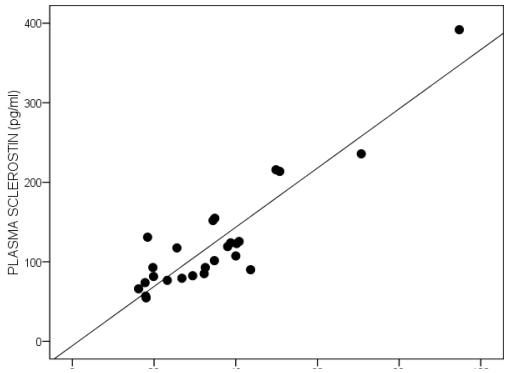


Figure 5. Relationship between plasma (EDTA) and serum levels of sclerostin in blood samples of 26 subjects (r=0.91, p<0.001). Sclerostin levels were 3.6 ± 1.0 times higher in plasma compared to serum samples.

Finally, to assess the day to day variation in sclerostin levels in an individual, we obtained non-fasting serum samples from 7 healthy subjects at the same time of the day on 3 separate occasions, at 3 to 4 day intervals. All samples were measured in the same run. Individual sclerostin levels at all 3 time points are depicted in Figure 6. Within subject variation, described as the coefficient of variation (standard deviation/mean*100) of 3 measurements, ranged between 9% and 29 % (mean 19%).

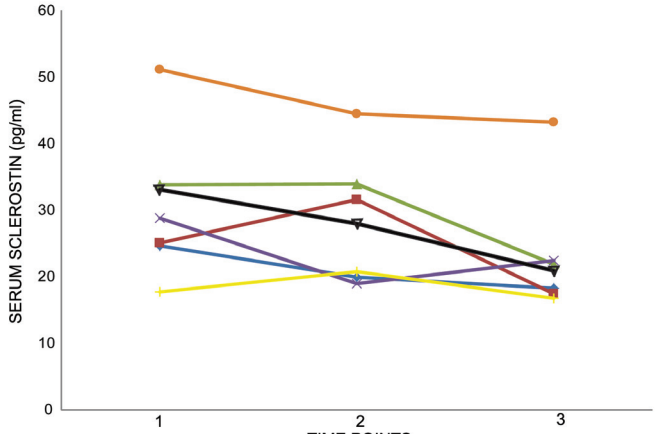


Figure 6. Within subject (n=7) variation in circulating sclerostin levels
Blood samples were collected at 3 different time point, 3 to 4 days apart.
Mean coefficient of variation was 19%

Conclusions

The epitopes of the capture and detection antibodies, and the absence of detection of sclerostin fragments of different length by the MSD sclerostin assay, suggests that this assay is specific for the entire sclerostin molecule, while the undetectable levels in all tested patients with sclerosteosis underscores its specificity for sclerostin and rules out interference of other proteins in the assay. The assay has a good precision and a wide detection range suitable for use in clinical studies. Furthermore, repeated freezing and thawing of samples did not change measured values suggesting that the molecule is stable. However, if samples remain at room temperature beyond 8 hours lower values may be measured. There is a clear difference in measured sclerostin values in serum and EDTA plasma. Although values in serum and plasma were highly concordant this should be considered in clinical studies and a new reference range should be obtained for plasma values (see Chapter 6). Finally, the large intra individual variation (19%), should be considered in studies evaluating sequential changes of circulating sclerostin in humans.

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Chapter 3

Patients with sclerosteosis and disease carriers: human models of the effect of sclerostin on bone turnover.

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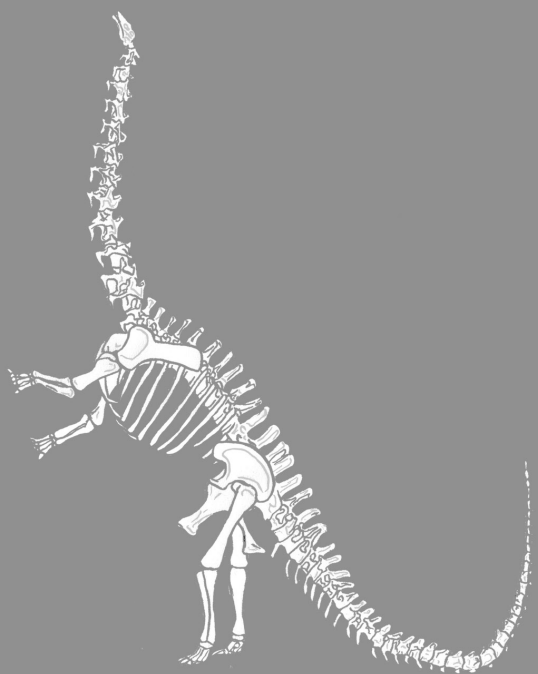

J Bone Miner Res. 2011 Dec;26(12):2804-11

Abstract

Sclerosteosis is a rare bone sclerosing dysplasia, caused by loss-of-function mutations in the *SOST* gene, encoding sclerostin, a negative regulator of bone formation.
The purpose of this study was to determine how the lack of sclerostin affects bone turnover in patients with sclerosteosis, and to assess whether sclerostin synthesis is decreased in carriers of the *SOST* mutation, and if so, to what extent this would affect their phenotype and bone formation. We measured sclerostin, P1NP, and CTX in serum of 19 patients with sclerosteosis, 26 heterozygous carriers of the C69T *SOST* mutation, and 77 healthy controls. Chips of compact bone discarded during routine surgery were also examined from 6 patients and 4 controls. Sclerostin was undetectable in serum of patients, but was measureable in all carriers (mean:15.5pg/ml; 95%CI:13.7-17.2pg/ml), in whom it was significantly lower than in healthy controls (40.0pg/ml; 36.9-42.7pg/ml; $p<0.001$). P1NP levels were highest in patients (153.7ng/ml;100.5-206.9ng/ml; $p=0.01$ vs carriers, $p=0.002$ vs controls), but carriers also had significantly higher P1NP levels (58.3ng/ml; 47.0-69.6ng/ml) than controls (37.8ng/ml; 34.9-42.0ng/ml;$p=0.006$). In patients and carriers, P1NP levels declined with age, reaching a plateau after the age of 20 years. Serum sclerostin and P1NP were negatively correlated in carriers and age- and gender-matched controls ($r= 0.40$, $p=0.008$). Mean CTX levels were well within the normal range and were not different between patients and disease-carriers after adjusting for age ($p=0.22$).
Our results provide in vivo evidence of increased bone formation caused by the absence or decreased synthesis of sclerostin in humans. They also suggest that inhibition of sclerostin can be titrated, since the decreased sclerostin levels in disease carriers did not lead to any of the symptoms or complications of the disease but had a positive effect on bone mass. Further studies are needed to clarify the role of sclerostin on bone resorption.

Introduction

Sclerosteosis is a rare, autosomal recessive bone sclerosing dysplasia characterized by generalized osteosclerosis (1). It is caused by loss-of-function mutations in the SOST gene encoding for sclerostin (2-4), a protein produced in bone by osteocytes (5) which decreases bone formation by inhibiting the Wnt signalling pathway in osteoblasts (6,7). In patients with sclerosteosis the lack of sclerostin leads to unrestrained bone formation resulting in generalized osteosclerosis.

The clinical manifestations of sclerosteosis have been well described since it was first identified in 1958 (8) and include cranial nerve deficits and increased intracranial pressure due to excessive growth of the skull bones (9,10). Bone mineral density (BMD) is markedly increased at the spine and the hip (11) but data on the rate of bone turnover are scarce in these patients. Interestingly, heterozygous carriers of the SOST mutation have high normal or increased BMD (11), suggesting that having one affected allele of the SOST gene would also have an effect on sclerostin synthesis, albeit milder than was observed in the homozygous state. Whether this may also be associated with clinical manifestations has not been studied systematically.

To address these questions we conducted a study in a cohort of patients with sclerosteosis and their relatives who were heterozygous carriers of the SOST mutation, with the following specific aims: first, to determine how the lack of sclerostin affects parameters of bone turnover in patients with sclerosteosis, and second to assess whether sclerostin synthesis is decreased in carriers of the SOST mutation and if so, to which extent this would affect their phenotype and bone turnover.

Subjects and Methods

Nineteen South African patients with sclerosteosis and 30 mostly first degree relatives living within a 250 km radius from Johannesburg were invited to participate in our study. None of the patients or relatives studied used any drugs that could affect bone metabolism. One patient was on long-term treatment with thyroid replacement therapy for hypothyroidism.

Physical examination, focusing on clinical features of sclerosteosis and on neurological deficits, was conducted at the Ear-Nose-and-Throat Department of the Flora Clinic in Johannesburg, or at the patients' home by A.v.L. and H.H. Since patients with

sclerosteosis are descendants of Dutch settlers, and because South African normative data are lacking, height z-scores were calculated relative to Dutch normative data, using Growth Analyzer 3.5 (Dutch Growth Foundation, Rotterdam, Holland). Results of hearing tests were obtained from the patient's hospital records. Non-fasting blood samples were collected as convenient, but samples from patients and their family members were collected at the same time of the day. Full blood samples and separated serum and plasma were frozen and transported to the Netherlands on dry ice for biochemical analysis and DNA extraction.
The study was conducted according to the principles of the Declaration of Helsinki, was approved by the Medical Ethical Committee of the Leiden University Medical Center and informed consent was obtained from all subjects included in the study.

DNA analysis
DNA was isolated from full blood samples using the Insorb® spin blood maxi kit (Invitek GmbH, Berlin, Germany). The first exon of the SOST gene was replicated by PCR, using primers 5'-AAGGAACTTGCCCAAGATGA-3' and 5'-AAGGCTCGAGCCCAAGATGA-3'. PCR products were purified with microspin^{tm} S-400 HR columns (GE Healthcare, Buchinghamshire, UK) and sent to the Leiden Genome Technology Centre for sequencing. DNA sequences were analysed with Chromas 2.33 (Technelysium Pty Ltd), focusing on the presence of a single cysteine to tyrosine substitution 69 base pairs downstream of the predicted translation initiation site (C69T), previously found to be the underlying mutation in South African patients with sclerosteosis (3).

Serum Biochemistry
All biochemical measurements were performed at the Clinical Chemistry Laboratory of the Leiden University Medical Center, Leiden. Serum calcium adjusted for albumin, phosphate, and creatinine were measured by semi-automated techniques. Alkaline phosphatase (ALP) was measured using a fully automated P800 modulator system (Roche BV, Woerden, Holland). P1NP and β-CTX were determined by the E-170 system (Roche BV, Woerden, Holland). 25-hydroxyvitamin D (25-OHD) was measured by the LIAISON® 25-OH Vitamin D TOTAL assay (DiaSorin S.A./N.V., Brussels, Belgium).

Sclerostin assay

Sclerostin was measured in serum by an electrochemiluminescense assay (MSD® 96-well MULTI-ARRAY® Human Sclerostin Assay, Meso-Scale Discoveries, Gaithersburg, Maryland, USA) as described previously (12). The assay is very sensitive (detection limit: ±1pg/ml), with a broad detection range (1 to 10,000 pg/ml), and an intra-assay precision of 6% and an inter-assay precision of 10%. The recovery of sclerostin in serum spiked with sclerostin 250, 125, and 65 pg/ml was 82%-93%.

Sclerostin is a glycoprotein containing 190 residues, which form a three loop-like structure, with a cystine knot at the base and long, highly flexible C- and N-terminal regions (13). Bioavailability of circulating form(s) of sclerostin is currently unknown and the specificity of commercial assays for different domains of the molecule has not been reported. We performed, therefore, additional experiments to obtain more insight into the specificity of the MSD assay for sclerostin.

First, to validate the sclerostin standard used in this assay, we compared it to that of recombinant sclerostin prepared by the University of Würzburg, Germany, kindly provided by Professor Thomas Mueller within the TALOS research consortium. This recombinant sclerostin was produced in an E. Coli strain, and the protein concentration was accurately determined by spectrophotometry. Serial dilutions (10 to 1000pg/ml) of both these sclerostin preparations were measured. Values of the two peptides were highly concordant at every concentration. The mean ratio of the concentrations was 1.02 (range 0.8-1.3). Second, to characterize the specificity of the antibodies used in the assay, we performed epitope mapping of the polyclonal detection and capture antibodies according to Pepscan's Epitope Mapping Technology [kindly performed by Jaap Willem Back and Peter Timmerman of Pepscan Therapeutics (Lelystad, the Netherlands) within the TALOS research consortium]. Pepscan's Epitope Mapping Technology uses microarrays of overlapping peptides, covering the complete sequence of a given protein. Antibody binding studies subsequently identify the peptides representing the protein interaction of interest. (http://www.pepscantherapeutics.com/technology/epitope-mapping). The epitope mapping revealed two binding sites of the capture antibody for human sclerostin, one on the N-terminus and one on the distal end of the C-terminus. For the detection antibody there were three apparent epitopes; at the N-terminus, at the 3rd loop, and at the C-terminus of the protein. We further validated these results by assessing reactivity of sclerostin fragments of

different sizes (provided by Jaap Willem Back of Pepscan therapeutics). We tested three different fragments comprising the three loops without the N- and C-terminus; the first and third loop without C- and N-terminus; and the second loop alone. All fragments were added at high concentrations (10 ng/ml). As was expected from the epitope mapping, all fragments were undetectable, suggesting that the assay detects the whole sclerostin molecule.

Serum sclerostin, and bone turnover marker values, were compared with those obtained in a cohort of healthy volunteers, who were used as controls as previously described (12). This group consisted of 77 healthy volunteers, 30 males and 47 females, with mean age 50.3 years (range: 20-77 years), and mean BMI of 25.2 kg/m^2. All had normal serum calcium and phosphate concentrations, normal renal function and biochemical markers of bone turnover.

Bone biopsies

Chips of compact bone, obtained from 6 patients with sclerosteosis (4 reported here), on a different occasion during surgical procedures, and from 4 controls subjects were examined. The samples were fixed in 10% neutral buffered formalin and were embedded in methyl methacrylate without prior decalcification (14,15). Ten micron sections were cut by a Jung Polycut E microtome (Leica, Milton Keynes, UK) and stained using solochrome cyanine R and Goldner's protocols (15). Images for the quantification of bone remodeling parameters were acquired under bright field illumination at x5 objective magnification using Surveyor software (Objective imaging Ltd, Cambridge, UK). The samples were essentially compact bone which was being remodeled in a manner similar to cortical bone, so that assessment of bone formation and resorption was done in the same way as that reported by Bell and colleagues (15). Canals within the bone were denoted as undergoing formation (presence of an osteoid seam) or resorption (presence of a crenelated surface) and the data were expressed as a proportion of the total number of canals within the specimen.

Statistical analysis

Statistical analysis was performed using the SPSS 17.0 software (SPSS Inc. Chicago, USA). Group differences in levels of sclerostin and other biochemical markers were assessed by ANOVA. Because of inequality in sample sizes, a Games-Howell post-hoc test was used. To adjust for effect of age on P1NP levels, we also matched carriers to

healthy controls according to gender and age (within five years). Correlations between sclerostin and bone turnover markers were determined by Pearson's correlation tests. P1NP and Sclerostin data were log transformed because of skewness. A p-value below 0.05 was considered significant.

Results

Subjects

The diagnosis of sclerosteosis was confirmed in all 19 patients studied by DNA analysis, on the basis of the demonstration of a C69T substitution in both alleles of the SOST gene. Of the 30 relatives, 26 were heterozygous carriers of the C69T mutation and were included in the analysis. Median age of the patients was 23 years (range 9 to 70 years), and of the carriers 44 years (range 13 to 70 years). Characteristics of patients and their heterozygous relatives are shown in Table 1.

Table 1. Characteristics and parameters of calcium metabolism in patients with sclerosteosis and heterozygous disease carriers

	Patients (n=19)	Carriers (n=26)	p value
male:female	11:8	7:19	0.16
Age (years)	28.32 ± 15.0	39.9 ± 15.0	0.014
Height (cm)	179.8 ± 12.0	164.0 ± 37.0	0.100
Height z-score (sd)	+0.83 ± 0.73	-0.48 ± 0.89	<0.001
BMI (kg/m²)	25.6 ± 5.7	25.7± 5.3	0.93
Calcium (mmol/l)	2.33 ± 0.07	2.27 ± 0.11	0.06
Phosphate (mmol/l)	1.37 ± 0.32	1.20 ± 0.32	0.10
25(OH)D (nmol/l)	48.9 ± 13.1	41.6 ± 15.0	0.10
Creatinine (μmol/l)	62.2 ± 20.4	55.2 ± 12.1	0.20

Values given as mean ± sd. BMI= body mass index; 25(OH)D= 25-hydroxyvitamin D

Clinical features

At the time of the study 3 of the 19 patients had symptoms related to increased intracranial pressure in the form of severe and persistent headaches, worse in the morning and associated with dizziness and nausea. Two of these 3 patients had already undergone decompressive surgery to relieve the increased intracranial pressure. Thirteen of the remaining 16 patients had undergone decompressive surgery, 10 after experiencing symptoms of increased intracranial pressure, and 3 following a diagnosis of increased intracranial pressure on routine screening. Decompressive surgery consisted of anterior, and/or posterior craniotomy, as described previously (16). The median age at which this procedure was performed was 15 years. Of the 7 patients who had undergone this procedure at a younger age, 4 (57%) had to be re-operated because of recurrence of signs of increased intracranial pressure after 2 to 11 years. Of the 8 patients in whom the operation was performed after the age of 15 years, only 1 (13%) patient had to be re-operated after an interval of 2 years.

The majority of patients (89%) had experienced recurrent episodes of facial palsy, usually occurring before the age of 4 years, although unilateral facial paresis was already present at birth in one patient. Surgical decompression of the facial nerve was conducted in all cases, with the unaffected side also decompressed prophylactically. Despite these interventions, facial palsy recurred in 6 of the previously operated cases. Hearing loss was present in all cases, had been recognized in early childhood and progressed into adulthood. Operations to improve hearing, such as widening of the external bony ear canal, or freeing of fixed ossicles, had been performed in 13 patients. Eleven patients used hearing aids. Other complaints associated with cranial nerves compression were decreased sensation of the face (trigeminal nerve) in two cases, and a visual field defect in one eye in one patient (optic nerve). None of the patients reported sustaining a bone fracture.

Overview of the medical history of the whole group provided insight into the natural history of the disorder. Disease manifestations first appear during childhood and adolescence and progress through to the third decade of life appearing to stabilize thereafter. In the majority of patients no recurrence or progression of symptoms were observed after the age of 25 years.

On clinical examination, the majority of patients were of tall stature. The average height of adult male patients was 190.6 cm, and of adult females 175.7 cm. Mean weight and body mass index (BMI) were respectively 104.4 kg and 28.8 kg/m^2 for

adult males, and 73.7 kg and 24.7 kg/m² for adult females. Mean height z-score of patients was above zero, and was significantly higher than that of their relatives ($p<0.001$) (Table 1). Patients had a sclerosteosis phenotype, as previously described, of variable degree of severity. The facial deformities of bossing of the forehead and enlargement of the mandible were observed in 47% and 68% of subjects, respectively, and had generally developed by the time puberty was reached. Although these features were not present in the two youngest patients, both 9 years old, they were already clearly noticeable in 2 young male patients, aged 12 and 13 years old. Six patients had undergone corrective surgery of the mandible. One of these patients needed a second corrective surgery 7 years later due to further enlargement of the mandible. Syndactyly of fingers or toes was present in 52% of the cases. All but one of the remaining patients did, however, display other digit abnormalities, such as nail dysplasia or radial deviation of the phalanges.

On neurological examination movement of the facial muscles was impaired in 73% of patients, bilateral in 52% of these cases, and of moderate (House Brackmann score: Grade III) to moderately severe (Grade IV) degree. Previously performed hearing tests were abnormal in all patients, showing conductive hearing loss of a moderately severe (50-70 dB) to severe (70-90dB) grade in all, with an additional sensorineural component in 36 % of the cases. In 2 patients hearing loss was complete. Cognitive function was normal in all patients.

None of the carriers reported having any of the above mentioned symptoms and none had abnormal findings on clinical examination. Similar to patients, none of the studied carriers had ever sustained a fracture.

Serum chemistry

There was no difference in serum calcium, phosphate and 25(OH)D concentrations between patients and carriers, and all had normal renal function (Table 1).

Markers of bone turnover

Serum P1NP levels declined with age in both patients and carriers and appeared to reach a plateau after the age of 20 years (Figure 1). Thirteen out of 14 adult patients (18 years or older) and 7 out of 22 adult carriers had serum P1NP values above 65 ng/ml. Because of the clear effect of age on serum P1NP levels and the low number of young individuals in the carrier group, we compared serum P1NP between

adult patients and carriers. P1NP levels were significantly different between groups (ANOVA: p<0.001). Compared with carriers and controls, patients with sclerosteosis had significantly higher serum P1NP values (153.7 ng/ml; 95%CI=100.5-206.9ng/ml; p=0.01 vs carriers, p=0.002 vs controls) while carriers (58.3 ng/ml; 95%CI=47.0-69.6 ng/ml) had significantly higher values than controls (37.8 ng/ml; 95%CI=34.5-41.0 ng/ml; p=0.006) (see also Figure 2) These differences remained after adjusting the P1NP values for age.

Similar to serum P1NP, serum CTX values declined with age reaching a plateau around the age of 20 years (Figure 1). However, in all but one adult patient values were lower than 600 pg/ml. There was a difference in mean serum CTX between adult patients (213 pg/ml; 95%CI=103-323 pg/ml) and carriers (126pg/ml; 95%CI=84-167pg/ml, p=0.02), but this was no longer significant after adjusting for age (p=0.22). Although absolute values for serum CTX should be interpreted with caution because not all samples were obtained in the fasting state, comparison of serum CTX between groups is valid, because samples were obtained from patients and carriers under identical conditions. Serum P1NP and CTX values were significantly correlated both in patients (r=0.86, p<0.001) and carriers (r=0.46, p=0.025).

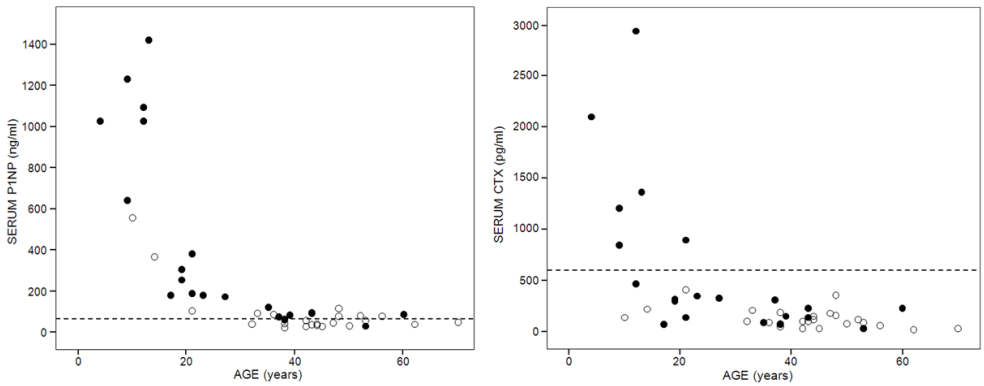

Figure 1. Relationship between serum P1NP levels and age (left panel) and CTX levels and age (right panel) in patients with sclerosteosis and heterozygous disease-carriers.
Closed circles represent homozygous patients, open circles represent heterozygous carriers.
The dotted lines represent the upper limit of the normal adult reference range
(65ng/ml for P1NP, 600pg/ml for CTX).

Serum sclerostin

Serum sclerostin was undetectable in all 19 patients. In contrast, sclerostin was measurable in the serum of all carriers, although the mean value (15.5 pg/ml; 95% CI=13.4-16.9pg/ml) was significantly lower than that of healthy controls (40.0 pg/ml; 95%CI=37.2-42.9 pg/ml; $p<0.001$) (Figure 2), and the difference remained significant after adjusting values for age. There was no correlation between serum sclerostin and either P1NP or CTX in carriers. However, when values of carriers and controls were pooled together, there was a significant negative correlation ($r=-0.23$, $p=0.02$) between sclerostin and P1NP, which improved further ($r=-0.40$, $p=0.008$) when carriers were analysed together with age and gendermatched controls (Figure 3).

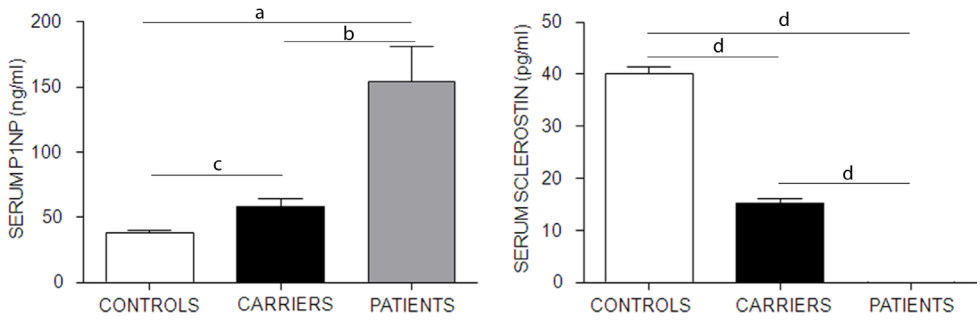

Figure 2. Serum P1NP (left panel) and sclerostin (right panel) levels in healthy controls and adult patients with sclerosteosis and heterozygous disease-carriers.
Bars represent SEM. a: p=0.002; b: p=0.01; c: p=0.006; d: p<0.001

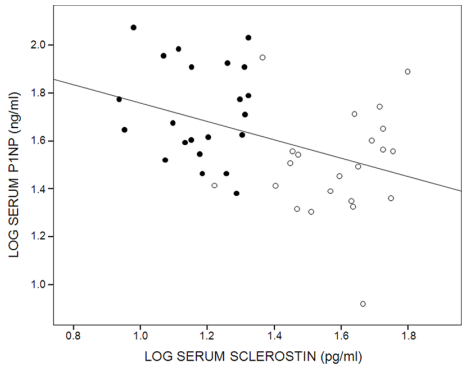

Figure 3. Relationship between serum P1NP and sclerostin levels in age-matched healthy controls (open circles) and heterozygous carriers of sclerosteosis (closed circles). r=-0.40, p=0.008

Histomorphometric analysis of bone remodeling

The results of histomorphometric analysis of bone samples are shown in Table 2. Bone from patients with sclerosteosis showed evidence of increased formation in that there was a higher proportion of canals with an osteoid seam compared to bone from similar sites in control subjects (sclerosteosis: 2.6 to 21.1%; controls: 1.1 to 3.7%; Table 2). The proportion of canals undergoing resorption was similar in both groups (sclerosteosis: 0.6 to 3.0%; controls 0 to 0.9%). In the patients, as with the relationship between serum P1NP and age, bone formation activity appeared to reach a peak at puberty. Direct comparison of histomorphometric values is, however, not appropriate due to differences in age between patients and controls.

Table 2. Histomophomety of patients with sclerosteosis and controls

Biopsy	Source	Age	Gender	Formation (Active/Total)	Resorption (Active/Total)
Controls					
1	Mastoid	37	Female	1/41 (2.4%)	0/54 (0.0%)
2	Mastoid	49	Female	1/37 (2.7%)	0/37 (0.0%)
3	Mastoid	49	Male	1/94 (1.1%)	0/113 (0.0%)
4	Mastoid	64	Male	4/108 (3.7%)	1/117 (0.9%)
Patients					
1	Mastoid	4	Male	8/311 (2.6%)	2/347 (0.6%)
2	Mastoid	8	Female	49/654 (7.5%)	9/686 (1.3%)
3	Mastoid	8	Male	10/66 (15.1%)	1/68 (1.5%)
4	External Hearing Canal	14	Male	11/52 (21.1%)	2/67 (3.0%)
5	Mastoid	18	Male	7/49 (14.2%)	1/47 (2.1%)
6	Mastoid	43	Male	8/77 (10.4%)	4/107 (0.9%)

Discussion

Patients with sclerosteosis and heterozygous disease carriers form a unique model to study the role of sclerostin on bone metabolism in humans. In this study we show that there is a gene-dose effect of the slerosteosis mutation on circulating sclerostin, with absent sclerostin in serum of patients and decreased sclerostin levels in disease carriers. These differences in circulating sclerostin were accompanied by different levels of the bone formation marker serum P1NP between patients, carriers and controls.

The recognition of the role of sclerostin in bone metabolism and the development of commercial assays for measuring it in serum, have led to a series of studies that explored the association between serum sclerostin and indices of bone metabolism in healthy individuals and in patients with various bone disorders (12,17-23). Caution is needed, however, in interpreting the results of these studies and in deriving conclusions about the pathophysiological significance of observed associations. Sclerostin is produced in bone by osteocytes, acts on osteoblasts and is released in the circulation. At present, neither the bioactivity of circulating sclerostin nor the specificity of the antibodies used in existing assays for the protein are known. Moreover, there are differences in measured values, and reported values obtained with different assays are poorly correlated (24). In the present study we used a highly sensitive assay with a very low limit of detection and we assessed for the first time its specificity in detecting sclerostin. Our findings suggest that this assay detects the whole sclerostin molecule rather than circulating fragments, which may be active. The lower values measured with this assay compared with those reported with other assays, may be attributed to lower detection of protein-bound forms of the protein.

Previous studies have shown that sclerostin is not expressed by osteocytes of patients with sclerosteosis (5) and our data on serum sclerostin are in agreement with these findings and the pathogenesis of the disease. We could not detect sclerostin in the serum of any patient with sclerosteosis, which provides a powerful negative control for the assay we used, supporting its specificity, and suggesting that measured values can be of biological significance. Contrary to patients with sclerosteosis, sclerostin was detectable in the serum of all carriers of the disease being, however, on average 60% lower than values measured in healthy controls. These lower circulating levels

of sclerostin most likely mirror a decreased synthesis of the protein by the osteocytes of these individuals as a result of the affected SOST allele. The reduced synthesis of sclerostin in disease carriers affects bone metabolism but is not associated with any of the clinical manifestations or complications of sclerosteosis. The clinical presentation of our patients with sclerosteosis is in accordance with earlier descriptions of the disease and confirms the high frequency of serious complications resulting from entrapment of cranial nerves due to increased bone formation in the skull. Disease carriers, however, were symptom-free and there were no clinical signs suggestive of any of these complications. This may be due to the lower rates of bone formation of carriers compared with patients as evidenced by serum P1NP values. On the other hand, when compared with healthy individuals, disease carriers had higher P1NP values and there was a significant negative correlation between serum sclerostin and P1NP values. These results help to explain the previously reported high BMD values of carriers (11) and minor changes apparent on skull radiographs (25). In addition, they suggest that non-excessive inhibition of sclerostin production may have a positive effect on the skeleton without causing any of the complications associated with the absence of the protein. Currently inhibitors of sclerostin are being developed as potential bone forming treatment for patients with osteoporosis, but values of circulating sclerostin and P1NP were not reported (26,27). Our data indicate that decreasing the synthesis of sclerostin can have a beneficial effect on the strength of the skeleton, as already shown in animal studies (28-30).

An important finding, as also previously noted by Beighton et al. (10), was the stabilization of the disease and the dramatic decrease in the frequency of complications after the third decade of life suggesting that the rate of bone formation slows down in patients with ageing. This hypothesis is supported by the negative correlation between age and serum P1NP values and by the histological findings. It is also of interest to note that serum P1NP changes follow a normal pattern during growth being high in childhood and adolescence and reaching a plateau after completion of growth. In addition, the highest bone formation rate, as assessed by histomorphometry, was found in a 14-year old patient. In a previous longitudinal study of a patient with the closely related bone dysplasia, van Buchem disease, we showed that biochemical markers of bone turnover were always increased for age but followed a normal pattern during growth with the highest levels observed during the growth spurt with a decline

thereafter (31). Taken together, these observations strongly suggest that the skeleton of patients lacking sclerostin responds normally to local and systemic signals, as also evidenced by the significant relationship between serum P1NP and CTX values. The lack of sclerostin may be also responsible for the patients' tall stature. Although this has been mentioned as a clinical feature of the disease, our study is the first to examine this in detail. Sclerostin is expressed by terminally differentiated chondrocytes (32,33), which can be regarded as the equivalent to osteocytes in the chondrocyte lineage, and canonical Wnt signaling promotes differentiation and maturation of chondrocytes (34). It might, therefore, well be that sclerostin has a similar inhibiting role on Wnt signaling in chondrocytes in the growth plate. In sclerosteosis the sclerostin deficiency would lead to increased differentiation towards hypertrophic chondrocytes resulting in a larger hypertrophic zone in the growth plate and, therefore, more new bone accrual and more longitudinal growth.

While serum P1NP levels were higher in patients with sclerosteosis compared with disease carriers, CTX levels, in non-fasting blood samples taken at the same time of the day, did not differ between these two groups. In a previous report another biochemical marker of bone resorption, hydroxyproline, was found to be within the normal range in 3 patients with sclerosteosis (35). These findings are in line with the histomorphometric results of the present study as well as with those in a murine model of the disease (36). In the latter, bone resorption as assessed biochemically and histologically, did not differ from that of wild type mice. Wnt signaling in osteoblasts decreases bone resorption by downregulating the expression of RANKL and upregulating that of OPG (37-39). In addition to stimulating bone formation, the lack of sclerostin leading to stimulation of Wnt signaling may, thus, also decrease the rate of bone resorption. This premise is supported by the changes in serum P1NP and CTX in animals and humans treated with an antibody to sclerostin (27,29).

The results of our study provide further strong evidence for the paradigm that inhibition of sclerostin activity has an anabolic effect on bone. Although patients with sclerosteosis and disease carriers form a proper model to study the effect of decreased sclerostin activity on bone in humans, this model differs from treatment with an inhibitor of sclerostin as in these individuals the decreased or absent sclerostin production is continuous and permanent while treatment with an inhibitor results in an immediate but reversible inhibition of sclerostin. The negative relationship

between serum P1NP and age we found in patients and disease carriers suggests that in sclerosteosis bone formation is not elevated to the same extent throughout life and that the absence of sclerostin alone does not act as a constant stimulus for bone formation. This raises the question whether prolonged treatment with a sclerostin inhibitor will be associated with a sustained anabolic effect on bone or whether the beneficial effect on bone formation may become blunted after a certain period of time. It should be noted that sclerostin does not stimulate osteoblastogenesis but rather acts at later stages of osteoblast development and inhibits their activity and reduces their life-span (5,32,40). In the young, osteoblastogenesis is increased as required for skeletal growth whereas it decreases after skeletal maturity. It may, therefore, be that in the presence of an increased pool of osteoblasts, as occurs in the young, the lack of sclerostin leads to excessive bone formation which, however, decreases considerably when this pool is reduced, as occurs in adults. The potential contribution to these responses of other inhibitors of the Wnt signalling pathway, such as Dkk1, warrants further investigation. This hypothesis can explain the changes of bone formation with age in the studied individuals and suggests that the response to exogenously administered inhibitors of sclerostin may be more complex than that illustrated by short-term studies in animals and humans.

In conclusion, our findings provide compelling in vivo evidence of how the absence or decreased synthesis of sclerostin leads to increased bone formation in humans. Furthermore, inhibition of sclerostin can be titrated since the decreased sclerostin levels in disease carriers, did not elevate bone formation to the same extent as in patients with sclerosteosis and did not lead to any of the symptoms or complications of the disease but had a positive effect on bone mass. Further studies are needed to clarify the role of sclerostin on bone resorption.

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Chapter 4

Van Buchem disease: Clinical, biochemical and densitometric features of patients and disease carriers.

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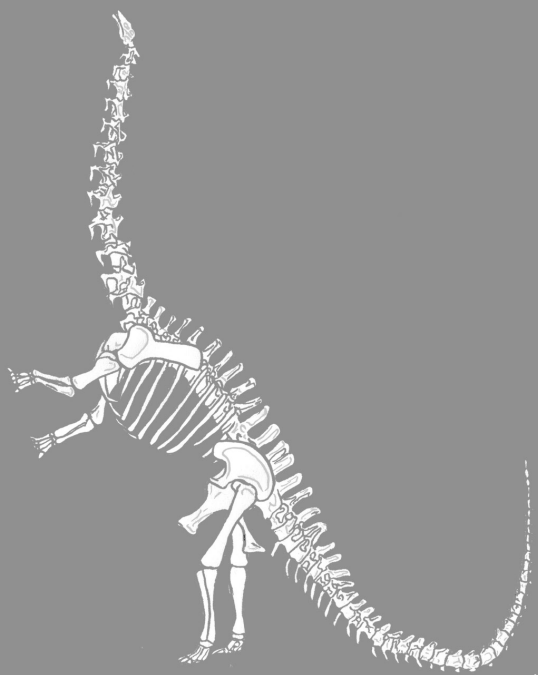

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Abstract

Van Buchem disease (VBD) is a rare bone sclerosing dysplasia caused by the lack of a regulatory element of the *SOST* gene, which encodes for sclerostin. We studied the demographic, clinical, biochemical and densitometric features of 15 patients with VBD (12 adults and 3 children) and 28 related carriers of the gene mutation.

The clinical course of the disease appeared to stabilize in adulthood with the majority of patients reporting no progression of symptoms or development of complications with time. Carriers of the disease had no clinical features or complications of the disease. Sclerostin could be detected in the serum in all but one patients [mean 8.0 pg/ml;95% Confidence Intervals (CI) 4.9-11.0 pg/ml], and were lower than those of carriers (mean 28.7pg/ml;95%CI 24.5-32.9pg/ml, $p<0.001$) and healthy controls (mean 40.0pg/ml;95%CI34.5-41.0pg/ml, $p<0.001$). Serum procollagen type 1 amino-terminal propeptide (P1NP) levels were also significantly higher in adult patients (mean 96.0ng/ml;95%CI 54.6-137.4ng/ml vs 47.8;95%CI 39.4-56.2ng/ml, $p=0.003$ in carriers and 37.8ng/ml;95%CI 34.5-41.0ng/ml, $p=0.028$ in healthy controls). Bone Mineral Density (BMD) was markedly increased in all patients (mean z-score 8.7 $\pm$ 2.1, and 9.5 $\pm$ 1.9 at the femoral neck and spine respectively); BMD of carriers was significantly lower than that of patients but varied widely (mean z-scores 0.9 $\pm$ 1.0 and 1.3 $\pm$ 1.5 at the femoral neck and spine, respectively). Serum sclerostin levels were inversely correlated with serum P1NP levels ($r=-0.39$, $p=0.018$) and BMD values (femoral neck $r=-0.69$, $p<0.001$; lumbar spine $r=-0.78$, $p<0.001$). Our results show that there is a gene-dose effect of the VBD deletion on circulating sclerostin and provide further in vivo evidence of the role of sclerostin in bone formation in humans. The small amounts of sclerostin produced by patients with VBD may explain their milder phenotype compared to that of patients with sclerosteosis, in whom serum sclerostin is undetectable.

Introduction

Van Buchem disease (VBD) or "hyperostosis corticalis generalisata familiaris" is a rare, autosomal recessive, bone sclerosing dysplasia, first described in 1955 (1). Another 13 cases were subsequently identified, all amongst inhabitants of a small village of the Netherlands (2, 3). The phenotype of patients with VBD is very similar to that of patients with sclerosteosis, a bone sclerosing dysplasia found mainly among the Afrikaners in South Africa (4). Both diseases are due to defects of the SOST gene, which is located on chromosome 17q12-q21 and encodes sclerostin, an osteocyte-produced negative regulator of bone formation (5). Whereas sclerosteosis is caused by homozygous mutations in the SOST gene, VBD is due to a homozygous deletion of a 52 kb regulatory element 35 kb downstream of the SOST gene, which leads to impaired production of sclerostin (6-9).

The clinical and biochemical features of patients with sclerosteosis and healthy disease carriers of this genetic abnormality have been well characterized (4, 10, 11), but less is known about VBD and carriers of the disease. Available data suggest clinical differences between the two sclerosing disorders (12) although information is lacking about potential biochemical differences between the two diseases and possible relationships between biochemical parameters and clinical and radiographic disease characteristics. The purpose of the present study was to characterize the demographic, clinical, biochemical and densitometric features of patients with VBD and of related carriers of the gene mutation, and to study the relationship between circulating sclerostin and bone turnover markers levels and BMD. A further aim of the study was to obtain insight in the natural course of VBD by retrospectively studying the clinical course of known patients with this disorder.

Subjects and Methods

Subjects

All known adult patients with VBD living in the Netherlands and their first degree relatives were invited to participate in this study. The majority of subjects were seen at their place of residence while those residing in other parts of the country were seen at the Leiden University Medical Center or at the University Medical Center Groningen.

A complete medical history was obtained from all patients, with special emphasis on complaints potentially associated with VBD. Physical examination including detailed neurologic examination and fundoscopy was performed in all patients but only when indicated in their relatives. Z-scores for height and head circumference were calculated and compared to the Dutch 1997 height for age-, and head circumference for age surveys using Growth Analyzer 3.5 (Dutch Growth Foundation, Rotterdam, the Netherlands).

Non-fasting blood samples were obtained from all subjects for DNA analysis and for the measurement of biochemical parameters of calcium and bone metabolism and of sclerostin. Subjects were seen at different times of the day, but patients and their relatives were seen at the same time on a particular day. Bone mineral density (BMD) of the spine and of the hip were measured by DXA (Lunar Prodigy, GE Healthcare, Hoevelaken, The Netherlands). Audiometry results and data from previous radiologic examinations were obtained from hospital records.
The study was approved by the Ethics Committee of the Leiden University Medical Center and written informed consent was obtained from all adult participants and from parents of children included in the study.

DNA analysis
DNA was tested for the presence of a homozygous or heterozygous 52kb deletion 35 kb downstream of the *SOST* gene on chromosome 17q12-q21, known to be the underlying genetic defect for VBD, in patients and carriers.

Serum Biochemistry
Blood was collected from all patients and heterozygous carriers and measured for serum calcium adjusted for albumin binding, phosphate, and creatinine, using semi-automated techniques. Alkaline phosphatase (ALP) was measured by a fully automated P800 modulator system (Roche BV, Woerden, The Netherlands). PTH was measured using the Immulite 2500 assay (Siemens diagnostics, Breda, The Netherlands). P1NP and β-CTX were determined by the E-170 system (Roche BV, Woerden, The Netherlands). 25-hydroxyvitamin D (25-OHD) was measured by the LIAISON® 25-OH Vitamin D TOTAL assay (DiaSorin S.A./N.V., Brussels, Belgium).

Serum Sclerostin

Sclerostin was measured in serum with an electrochemiluminescence assay (MSD® 96-well MULTI-ARRAY® Human Sclerostin Assay, Meso-Scale Discoveries, Gaithersburg, Maryland, USA) as previously described (11). Using this assay, we have previously shown that sclerostin was undetectable in the serum of all of 19 patients with sclerosteosis tested (11). The intra- and inter–assay coefficient of variation were 6% and 10%, respectively, and the detection limit was ±1 pg/ml. For comparison of sclerostin and P1NP levels we used as control a group of 77 healthy subjects, as reported in a previous study (13). Mean age of the control group was 50.3 years (range 20-77), and mean BMI was 25,2 kg/m^2 (range 19.0-36.5).

Statistical analysis

Statistical analysis was performed using the SPSS 17.0 software. Differences between groups were analyzed using ANOVA or Student's t-test as appropriate. A Games-Howell post-hoc test was used, because of inequality in sample sizes. Correlations between sclerostin, biochemical markers, age, and BMD values were assessed by Pearson's correlation testing. P1NP and CTX data were log transformed because of skewness. A p-value below 0.05 was considered to be statistically significant.

Results

I. Patients with VBD

Fifteen of the 18 known Dutch patients with VBD consented to participate in the study (12 adults and 3 children). The diagnosis was confirmed in all 15 patients by the presence of the VBD deletion on chromosome 17q12-q21. Of the 32 first degree relatives who participated in the study, 28 adults were found to be carriers of the disease and were included in the analysis. Demographic characteristics of patients and heterozygous carriers are shown in Table 1.

Clinical features

All 15 patients had experienced in the past one or more episodes of *facial palsy*. The median age at first occurrence was 2.5 years. Facial palsy was already present at birth in two patients, and was observed within the first year of life in another two. Unilateral or bilateral surgical decompression of the facial nerve was performed in 6 of the 15 patients. Five of the 15 patients reported recurrent episodes of ipsilateral facial palsy. Fourteen patients reported some degree of *hearing impairment*. In 6 of these, this preceded or occurred concurrently with a middle ear infection. The age at which hearing loss became noticeable differed markedly among patients, developing in early childhood in some, while only noticed in late adulthood in others. Five of the 15 patients used hearing aids and 6 had undergone previous surgery (mastoidectomy because of chronic otitis media, removal of exostosis from the auditory canal, placement of Bone Anchored Hearings Aids (BAHA)).No patient reported visual impairment. Two patients reported a decreased sense of smell. Three patients had previously experienced symptoms of *raised intracranial pressure,*. Following lumbar punction, symptoms completely resolved in one patient, while in another they improved over several months with concomitant treatment with acetazolamide. In the third patient a ventricular peritoneal drain had to be placed and treatment with prednisone was given leading to complete resolution of symptoms (14). One patient had sustained a *fracture* of the wrist on two separate occasions, one at the age of 14 years after falling from a tree, and the other at the age of 17 years after a motorcycle accident. No fractures were reported by any other patient, despite involvement of some in major accidents. All patients reported to have trouble keeping afloat in water, only able to keep from sinking by active and continuous swimming.

Table 1. Van Buchem disease: characteristics of patients and carriers of the disease

	Patients	Carriers	p-value[a]
Male: female	10:5	17:11	0.11
Age (years)	39.07 ±20.9	36.0±16.9	0.68
Height (cm)	182.9 ± 9.5	175.2 ± 8.2	0.02
Height (z-score)	0.31 ±0.81	-0.16 ± 0.88	0.12
BMI (kg/m^2)	27.1 ± 4.2	25.0 ± 5.8	0.33
BMD FN (g/cm^2)	2.16 ± 0.41	1.17 ± 0.16	<0.01
BMD FN z-score (sd)	8.7 ± 2.1	0.9 ± 1.0	<0.01
BMD spine (g/cm^2)	2.13 ± 0.54	1.31 ± 0.17	<0.01
BMD spine z- score (sd)	9.5 ± 1.9	1.3 ± 1.5	<0.01
PTH (pmol/l)	8.8 ± 5.9	5.7 ± 4.0	0.13
Calcium (mmol/l)	2.42 ± 0.22	2.45 ± 0.15	0.62
Phosphate (mmol/l)	1.12 ± 0.33	1.10 ± 0.22	0.96
25 OH D (nmol/l)	53.9 ± 20.3	58.4 ± 14.7	0.43
Creatinine (µmol/l)	77.5 ± 40.0	70.9 ± 12.1	0.53

Values are given as mean ± SD; [a] patients vs carriers; FN=Femoral Neck

The clinical course of the disease appeared to stabilize in adulthood with the majority of patients reporting no progression of symptoms or development of complications with time. Three patients (aged 49, 52 and 82) did, however, experience a further decrease in hearing with time. and one patient (age 43) reported continuing increase in the size of her lower jaw . None of the patients reported symptoms related to other organs such as heart, lungs, urogenital or gastrointestinal tracts, except for an 82 year old man, who had diabetes mellitus and heart failure associated with hypertension.

Clinical Examination

On clinical examination all patients had normal stature (Table 1) and all adults had some degree of facial distortion. Eleven of the 12 adult patients had a large, high forehead, and the mandible was enlarged in 10, with one patient having had corrective surgery of her lower jaw. The 3 children had no apparent facial dysmorphic features (15). The average circumference of the skull in adults was 63.6 cm (z-score:+3.4) for men, and 61.3 cm (z-score: +3.2) for women. In the children skull circumferences were 50.5 cm (z-score: 0), 49.8 cm (z-score: 0), and 52.3 cm (z-score: +1.3).

None of the patients had any abnormalities of hands or digits, such as syndactyly or nail hypoplasia. Blood pressure was normal in all but one patient and all had normal sinus cardiac rhythm.

On neurological examination none of the patients had anosmia, visual field defects or papilloedema, except for a boy aged 5 years. Oculomotor function was normal and pupil responses were adequate in all. Sensibility of the face was impaired in two patients. All patients had slight to severe facial palsy (House Brackman score II to V) which was unilateral in two and bilateral in the remaining 13 patients. On previous otoscopy, exostosis was observed in the bony part of the external auditory canal in 10 patients, in some of whom the external auditory canal was narrowed to a lumen of less than 2 mm. In one patient there was a complete fixation of all the ossicles of the middle ear. Hearing tests had been performed at some stage in all patients. In 3 patients there was no hearing loss, while this was mild in 3, moderate in 3, moderately severe in 2, severe in 1 and profound in 3. The type of hearing loss varied between patients, (sensorineural, conductive or mixed). Deep tendon reflexes were normal and all patients had normal plantar responses.

Bone Mineral Density

Bone mineral density (BMD) measured in 11 adult patients, and in 2 children, was increased in all (Table 1). BMD increased with age, but appeared to reach a plateau in late adulthood (Figure 1)

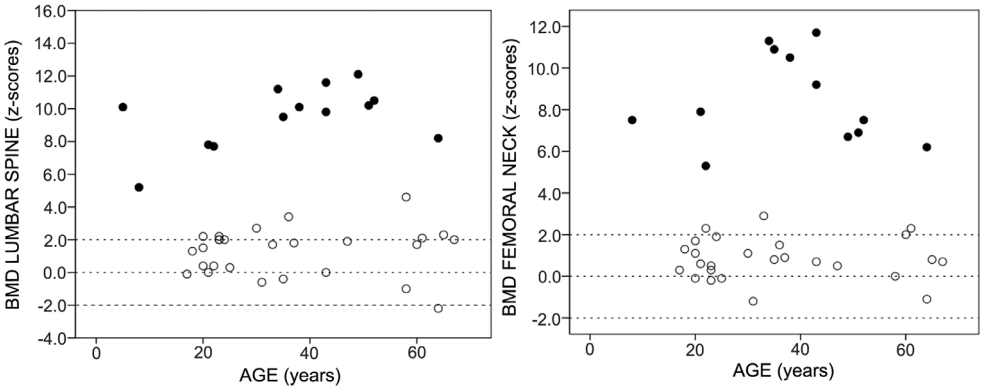

Figure 1. Bone mineral density (Z-scores) of the lumbar spine (left panel) and femoral neck (right panel) in patients with van Buchem disease (closed circles) and heterozygous carriers of the disease (open circles). Dotted lines represent -2, 0 and +2 standard deviations.

Radiology

CT scans of the mastoid, skull, and/or cervical spine had been performed in 9 patients. All scans showed a generalized increased thickness of all skull bones, with calvarial width measurements of greater than 2 cm in adult patients. A CT scan of the mastoid bones has been performed in 7 adult patients, in all of whom there was an evident narrowing or the internal auditory meatus. Narrowing of the internal auditory meatus could already be observed in a 3-year old, but absent in a 6-year old child. Narrowing of the facial nerve canal was observed in 3 patients. A cervical spinal stenosis due to hyperostosis of the vertebrae and to degenerative changes of the intervertebral discs was observed in 3 patients, in one of whom it resulted in a myelopathy. One patients had lumbar spine stenosis due to hypertrophic facet arthrosis and bulging of the intervertebral discs.

II. VBD disease carriers

Two of 28 studied heterozygous carriers had complaints potentially related to VBD. These 2 carriers, the mother and sister of a studied patient, had both sustained a transient unilateral facial palsy during labor which was quickly resolved. At the time of the study there were no residual neurological signs of facial paralysis in either. Six of the 28 carriers had sustained a fracture (upper arm 2, leg 2 , wrist 1, metacarpal

1), all, but one, having occurred in childhood, and all associated with appropriate trauma.

Physical examination of the 28 carriers was unremarkable. Height and BMI were normal and not significantly different from those of patients (Table 1). All subjects had normal blood pressure and sinus cardiac rhythm.

Mean BMD at the lumbar spine and the femoral neck are shown in Table 1. BMD values were significantly lower than those of patients but varied widely (Figure 1). z-scores ranged between -2.2 and +4.6 at the spine, and between -1.1 and + 2.9 at the femoral neck At the spine, 19 subjects (70%) had BMD z- scores above 0 with 6 subjects (22%) exceeding 2 SDs. BMD z-scores of the femoral neck were greater than 0 in 21 carriers (81%), exceeding 2 SDs in 3 subjects (12%).

Laboratory investigations

Blood was collected from 14 patients and 28 carriers and from 77 healthy controls for measurement of biochemical markers of calcium metabolism and bone turnover. Mean calcium, phosphate, 25-OHD, and PTH concentrations did not differ between patients and carriers (Table 1). There were no abnormalities in hematological parameters in the 7 patients in whom these were tested.

Serum P1NP levels declined with age in both patients and carriers (Figure 2). In adult patients, this was greater than 65 ng/ml in 67%. Serum P1NP was also increased in 19% of adult heterozygous carriers. Serum P1NP levels were significantly higher in adult patients with VBD than in carriers of the disease (96.0; 95%CI 54.6-137.4 ng/ml vs 47.8; 95%CI 39.4-56.2 ng/ml, p=0.003) and healthy controls (37.8; 95%CI 34.5-41.0, p=0.028) (Figure 3). There was no significant difference in serum P1NP values between carriers and healthy controls (p=0.14).

Serum levels of CTX, obtained in the non-fasting state, declined with age in patients, but not in carriers (Figure 2). These were higher than the upper limit of the normal reference range in 3 patients (25%) but in none of the carriers. Serum CTX levels were higher in patients (mean 447 pg/ml; 95%CI 266-628 ng/ml) compared to carriers

(mean 216 pg.ml; 95%CI 167-266 pg/ml) (p=0.020). Serum levels of CTX and P1NP were significantly correlated in both patients (r=0.95, p<0.001) and carriers (r=0.71, p<0.001).

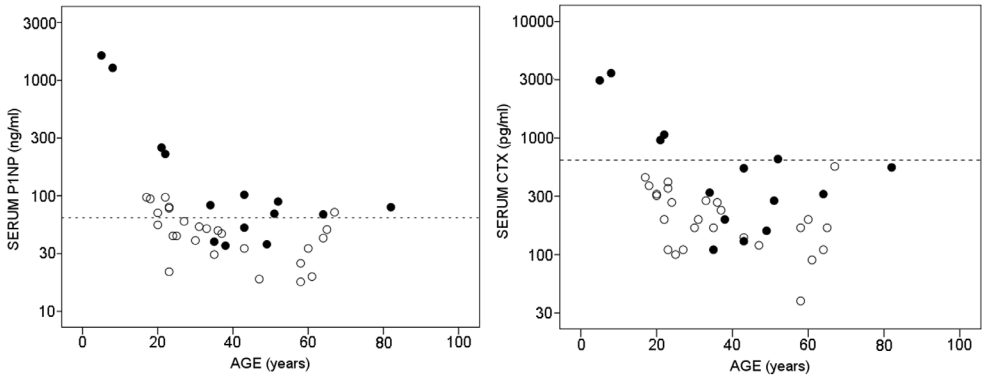

Figure 2. Relationship between serum P1NP levels and age (left panel) and CTX levels and age (right panel) in patients with van Buchem disease (closed circles) and heterozygous carriers of the disease (open circles). Dotted lines represent the upper limit of the normal adult reference range (65ng/ml for P1NP, 600 pg/ml for CTX)

Serum sclerostin

Sclerostin was detectable in the serum of all but one patient, and ranged between 1.7 pg/ml and 16.9 pg/ml. All carriers had detectable serum sclerostin levels with values ranging between 14.4 pg/ml and 55.0 pg/ml. VBD patients had significantly lower sclerostin levels (mean 8.0 pg/ml; 95%CI 4.9-11.0 pg/ml) compared to carriers (mean 28.7 pg/ml; 95%CI 24.5-32.9 pg/ml, p<0.001), and to healthy controls (mean 40.0 pg/ml; 95%CI 34.5-41.0 pg/ml, p<0.001). Mean sclerostin levels were also significantly lower in carriers than in controls (p<0.001) (Figure 3).

The relationship between serum sclerostin, bone turnover markers and BMD was studied by pooling data from all adult patients and carriers, in order to obtain a broader range of sclerostin values. In this pooled cohort, serum sclerostin levels were inversely correlated with serum P1NP levels (r=-0.39, p=0.018) (figure 4 A), but not with serum CTX (r=-0.28, p=0.11). Serum sclerostin levels were also inversely

correlated with BMD (g/cm²) at both the femur neck (r=-0.69, p<0.001) and the lumbar spine (r=-0.78, p<0.001).

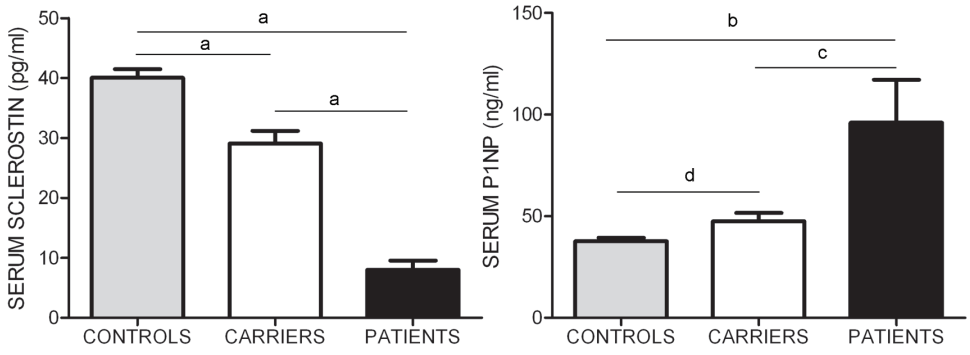

Figure 3. Serum P1NP (left panel) and sclerostin (right panel) levels in healthy controls, adult patients with van Buchem disease and heterozygous carriers of the disease. Bars represent SEM. a: p<0.001; b: p=0.003; c: p=0.028; d: p=0.13

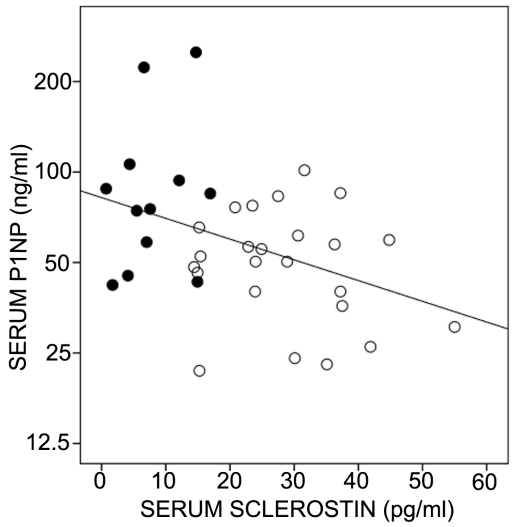

Figure 4. Relationship between serum P1NP and sclerostin levels in patients with van Buchem disease (closed circles) and heterozygous disease-carriers (open circles) r=-0.39, p=0.018

Discussion

Since the first description of VBD, approximately 30 cases have been reported, of which the vast majority is Dutch and living in the Netherlands. Before the discovery of the genetic background of the disease, a number of patients from the USA (16, 17), UK (18-20), and Italy (21) were diagnosed as having VBD, based on their clinical presentation. In some of these cases, however, there was an autosomal dominant inheritance (17, 19, 21), making it more likely that they suffered from a 'high bone mass disorder', or Worth's disease (22), caused by mutations in the LRP5 receptor as later confirmed by genetic analysis in an Italian family (23). However, two siblings with genetically confirmed VBD were recently reported from Germany, although no information about their genetic background were provided (24).

The phenotype of our patients with VBD is in keeping with previously described clinical features of the disease (1-3, 15, 22, 27). Our data also confirm that the clinical course of VBD is less severe than that of sclerosteosis, in which the majority of patients develop conductive hearing loss in early childhood and raised intracranial pressure around adolescence or early adulthood (4, 11), the latter being the main cause of death in patients with sclerosteosis. Raised intracranial pressure was much less frequently observed in patients with VBD (20%), and none of the 3 patients with documented increased intracranial pressure required a craniotomy, in contrast to the case in patients with sclerosteosis (25). There was, however, a large variation in the severity of clinical findings among patients but none of the measured laboratory parameters could explain these differences.

Facial palsy was a universal finding in studied patients. It developed early, at an age similar to that of patients with sclerosteosis, and was already present at birth in two of them. The reported occurrence of facial palsy at birth in a number of patients with VBD suggests that the fallopian canal may already be narrowed *in utero*. However, the regulatory element of SOST, which is lacking in patients with VBD, was not found to control embryonic SOST transcription (26), which might explain the absence of syndactyly and other digit abnormalities in VBD. Hearing loss was also common but less severe than in patients with sclerosteosis. These findings suggest that the extent of bone overgrowth during the first years of life might be similar in VBD and in sclerosteosis and progression later in life appears to occur more slowly in VBD than in sclerosteosis. Complications associated with bone overgrowth

appeared to stabilize after the third decade of life in both diseases (4, 11).
Consistent with the clinical findings and similar to the case in sclerosteosis (11), serum P1NP levels declined with age, reaching normal levels in several patients. This was consistent with no further increases in BMD. The stabilization of BMD we observed, contrasts with the findings of a previous study, in which the diaphyseal / metaphyseal ratio and the cortical thickness of metacarpals were reported to increase with age in patients with VBD, and in which an adult patient with progressive growth of the mandible was described (27). The oldest patient in our study, aged 82 years, had an enlarged mandible that demonstrated no progressive growth for decades, and progressive enlargement of the mandible has not been reported in patients with sclerosteosis.
VBD is caused by a deficiency of sclerostin synthesis. As shown here, all but one of the patients had detectable levels of sclerostin in serum which were, however, significantly lower than those of heterozygous carriers of the disease. These data contrast with findings in patients with sclerosteosis (11) in whom the *SOST* gene is affected, rendering the synthesis of sclerostin impossible. The regulatory element which is missing in VBD has been shown to be of crucial importance for the transcription of SOST in bone (26). It is, therefore, likely that in VBD, sclerostin production is due either to leaky transcription, the accidental transcription of a gene without prior activation of its regulatory element, or to the production of sclerostin by cells other than osteocytes in which this regulatory element is not essential. In favour of the first hypothesis is the finding of a weak sclerostin signal by immunological staining in a bone biopsy from a patient with VBD (28)Alternatively, circulating sclerostin might be secreted by cementocytes or hypertrophic chondrocytes (28, 29). We have previously shown that sclerostin is not expressed in cementocytes of patients with VBD (28) but there are no data about its potential production by chondrocytes in these patients. In contrast to patients with sclerosteosis who are tall, patients with VBD have normal height which is not different from that of carriers of the disease. We have previously speculated that the tall stature of patients with sclerosteosis might be due to lack of sclerostin at the growth plate which might not be the case in patients with VBD and at least part of the measured circulating sclerostin may originate from chondrocytes. Clearly more studies are needed to clarify this issue. Independently of these considerations, the milder clinical phenotype of patients with VBD compared to that of sclerosteosis is in line with the differences in sclerostin

levels between the two diseases.

As with sclerosteosis, a gene-dose effect was also present in VBD with patients having lower levels of sclerostin and higher levels of P1NP than their heterozygous carriers. These sclerostin levels were further significantly and negatively associated with BMD underscoring the importance of sclerostin in the process of bone formation. In line with the findings in patients, carriers of VBD had higher levels of serum sclerostin than carriers of sclerosteosis. Consequently, the difference in mean serum P1NP levels between VBD carriers and healthy controls was also smaller, and did not reach significance. There was also a large variation in BMD values in carriers with some individuals having very high values while others had low values, in contrast to the BMD of carriers of sclerosteosis which was found to be either high normal or increased, with none of the patients having low values (30).

Since its discovery a decade ago there has been much interest in sclerostin because of its key role in the regulation of bone formation and its use as target for new bone building therapies for osteoporosis (31). Our data provide further support to the rationale that sclerostin functions as a negative regulator of bone formation in humans. Not only had patients with VBD higher P1NP levels in comparison to carriers and controls, but sclerostin levels were also negatively associated with P1NP levels in a pooled cohort of patients and carriers.

The stabilization of the clinical course of VBD with time, and the decline in P1NP levels in patients with VBD and sclerosteosis with time raise the question whether sclerostin controls bone formation to the same extent throughout life. During childhood, longitudinal growth and strengthening of the skeleton by bone modelling requires high bone formation rates. We believe that sclerostin is extremely important during bone modelling to keep the vast numbers of active osteoblasts in check. Later in life, when the skeleton has matured, bone formation takes place as part of the bone remodeling process, and osteoblast number and activity is far less than that required for the process of bone modeling. Although sclerostin plays a clear role in the regulation of osteoblast activity during bone remodeling, sclerostin deficiency appears to be less important during this process than during bone modeling. It may, thus, well be that the absence of sclerostin during bone remodeling may be compensated to a certain extent by the action of other Wnt-antagonists, such as DKK1, or by other mechanisms maintaining the equilibrium between bone formation and resorption. This remains however speculative, and further studies are required to

examine this hypothesis.

Acknowledgements

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Chapter 5

Glucocorticoids are not always deleterious for bone

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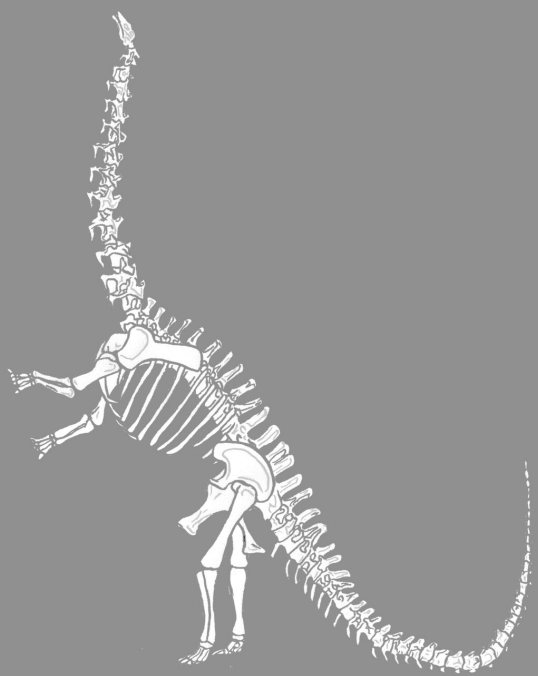
SE Papapoulos



J Bone Miner Res. 2010;25(12):2796-800

Abstract

A 25 year old man with the rare sclerosing bone disorder van Buchem disease presented with progressively worsening headaches eventually becoming persistent and associated with papilloedema. Increased intracranial pressure was diagnosed and the patient had a ventriculo-peritoneal drain inserted as well as simultaneously receiving treatment with prednisone. Before starting treatment, there was biochemical evidence for increased bone turnover and for steady increases in BMD at spine and total hip, despite the patient having reached his peak height of 197 cm at the age of 19 years. Treatment with prednisone resulted in biochemical and histological suppression of bone formation as well as of bone resorption, and in arrest of further bone accumulation.

Our data suggest that glucocorticoids (GC) may represent an attractive alternative to the high risk surgical approaches used in the management of patients with progressive sclerosing bone disorders. Our findings also suggest that whereas sclerostin may not be required for the action of GC on bone formation, it may well be important for the action of GC on bone resorption. The exact mechanism by which sclerostin may be involved in the regulation of bone resorption is as yet to be explored.

Introduction

Van Buchem disease is a rare bone sclerosing disorder described for the first time in 1955.(1) It belongs to the group of craniotubular hyperostoses and is characterized by progressive generalized osteosclerosis, particularly of the mandible and the skull, due to excessive bone formation.(2) It is caused by a 52kb deletion 35kb downstream the SOST gene which encodes sclerostin on chromosome 17q12-21.(3,4) This protein is produced in the skeleton exclusively by the osteocytes and inhibits bone formation by antagonizing the Wnt signaling pathway.(5) Clinical manifestations of the disease are due to entrapment of cranial nerves often associated with facial palsy, and loss of hearing and smell.(2) Van Buchem disease is thought to have milder clinical manifestations than sclerosteosis, a craniotubular hyperostosis with similar phenotype due to inactivating mutations of the SOST gene.(6,7) Management of the complications of both these sclerosing dysplasias is surgical, aiming at removal of the excess of bone, a technically difficult and sometimes dangerous procedure.(8,9,10) No medical treatment is available for either sclerosing diseases. Glucocorticoids are known inhibitors of bone formation(11,12) and we hypothesized that administration of these agents to patients with complications due to bone overgrowth may arrest its further progress.

We present here sequential observations of a patient with van Buchem disease with life-threatening increased intracranial pressure who was successfully treated with prednisone.

Case Report

The patient first came under our care at the age of 10 years with an established diagnosis of van Buchem disease. The disease was clinically and radiologically diagnosed in infancy and later genetically confirmed by the finding of a 52kb homozygous deletion 35 kb downstream the SOST gene on chromosome 17q12-q21 [the patient was briefly described (patient 15) by Staeling-Hampton et al(3)]. The parents are consanguinous and were both confirmed to be heterozygotes for the disease. There were 3 phenotypically normal sisters in whom no genetic testing has been so far undertaken.

As described in this disorder, clinical manifestations started early in childhood. The patient had a facial palsy at the age of 3 years and developed progressive deafness requiring the use of a hearing aid by the age of 10 years, followed by bilateral Bone Anchored Hearing Aids. He has been otherwise well with normal growth development along the 95th centile reaching a final height of 197 cm by the age 19 years. He completed his secondary education and is employed as office assistant manager. He married at the age of 20 years and he is father to 3 healthy children.
He demonstrated the typical clinical and radiological features of van Buchem disease, with enlarged head and mandible and no syndactyly or other digit malformations. During the 15-year duration of follow-up there were no other clinical signs or symptoms and blood pressure was normal. Haematological and biochemical parameters, including these of mineral metabolism demonstrated no abnormalities over the years. Skeletal radiographs showed thickening of the calvarium, base of the skull and long bones and sclerosis of the vertebrae (Figure 1). Bone Mineral Density (BMD) of the spine and hip were markedly increased at presentation (Z-score +6.2) and continued to increase in parallel with that of his healthy peers without, however, attaining a peak (highest z-score being 7.7). Biochemical markers of bone turnover were always increased compared to normal values for age, but followed a normal pattern of change with a further increase during the growth spurt and a progressive decline thereafter, although never reaching the normal range (Figure 2).

At the age of 23 years the patient complained of progressive headaches, eventually becoming persistent, associated with dizziness and by worse signs of increased intracranial pressure, in the form of papilloedema. The diagnosis was confirmed radiologically and a ventricular-peritoneal drain was implanted and he was concomitantly started on prednisone 30 mg/d which was reduced to 10 mg /d within one month. In the following 2 years he received different doses of prednisone, as depicted in Figure 3. These interventions were followed by rapid improvement of his symptoms which was sustained during the follow-up period.

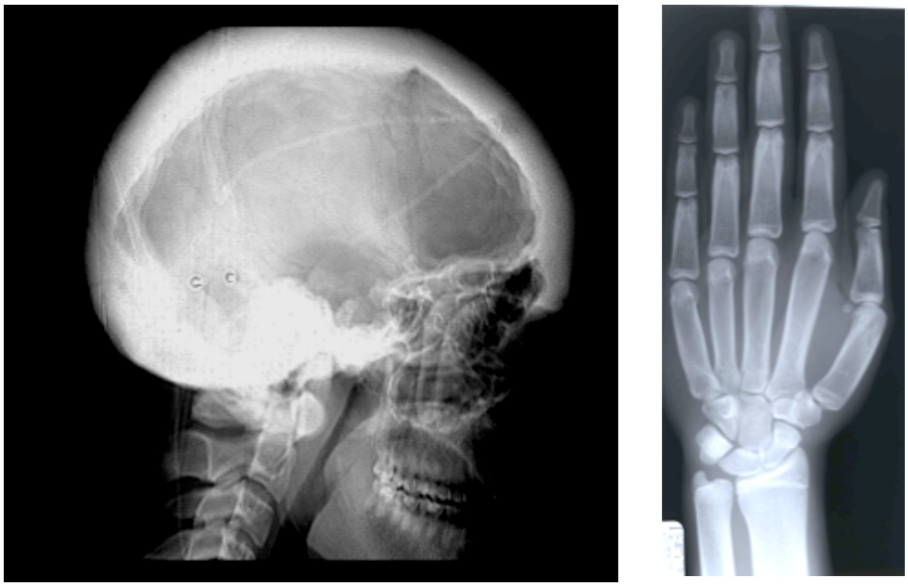


Figure 1: Radiographs of the skull, showing thickening of the calvarium and the base of the skull, and of the hand illustrating the absence of syndactyly or other malformations.

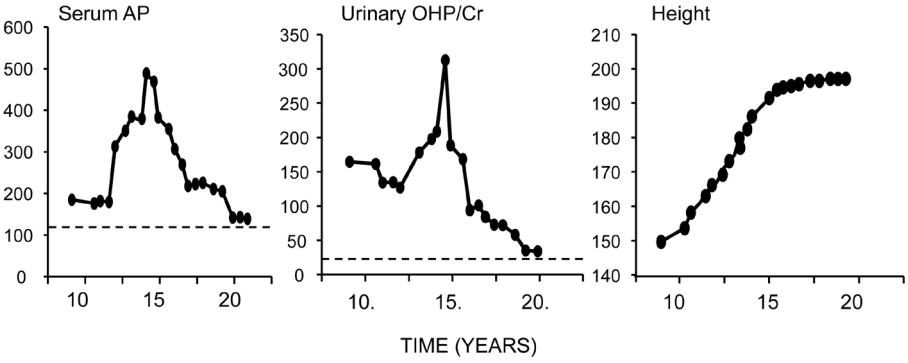


Figure 2. Sequential measurement of serum Alkaline Phosphate (AP) in U/l, urinary hydroxyproline to creatinine tario (OHP/Cr) in µmol/mmol and height (cm) in a patient with van Buchem disease over a 10-year period. Interrupted lines indicate the upper limit of the normal range.

Methods

The biochemical markers of bone turnover P1NP and β-CTX were measured in serum at regular intervals using the E-170 system (Roche BV, Woerden, Holland). BMD was measured by DXA (Hologic QDR 4500, Waltham, MA, USA). An iliac crest biopsy was obtained after in vivo labeling with two courses of tetracycline separated by 12 days. Bone histomorphometry was performed on undecalcified histological bone sections by Dr Pascale Chavassieux, INSERM Unit 831, University of Lyon, Faculty of Medicine R Laennec, France). Immunohistochemical staining for sclerostin was performed in our laboratory using a previously described technique.(13)

Results

Biochemical markers of bone turnover

The changes in serum P1NP and β-CTX before and during prednisone treatment are depicted in Figure 3. Before treatment, values of both markers of bone turnover were elevated and decreased to within the normal reference adult range within 4 weeks of starting treatment with prednisone. The effect of prednisone on bone turnover was dependent on the dose administered and attempts to reduce the dose below 5 mg/d were associated with increases in serum markers of bone turnover. It was notable that during treatment there was a close relationship between serum β-CTX and P1NP values, with the two markers demonstrating parallel changes during adjustments of the dose of prednisone, suggesting a tight coupling of bone resorption and bone formation. There was a highly significant correlation between the two markers throughout the 2-year period of follow up ($R2=0.765$).

BMD

The changes in BMD measured at the spine and at the hip for the 6 years preceding the start of prednisone treatment and for 2 years thereafter are shown in Table 1. Despite high baseline values, BMD continued to increase steadily during adulthood by about 4% per two years, demonstrating no further increase after two years of treatment with prednisone .

Bone histology

On an iliac crest biopsy, taken two years after the start of prednisone treatment, there was sclerosis and no evidence of active bone remodeling. Cancellous bone volume was clearly increased and bone trabeculae were thick and well connected. The extent of eroded surfaces was very low (0.4%; normal 3.1±1.1%) and Howship's lacunae were devoid of osteoclasts. In addition, no osteoid seams were seen and there was no tetracycline uptake upon examination under fluorescent light. As expected and previously described,(14) osteocytes did not stain for sclerostin.

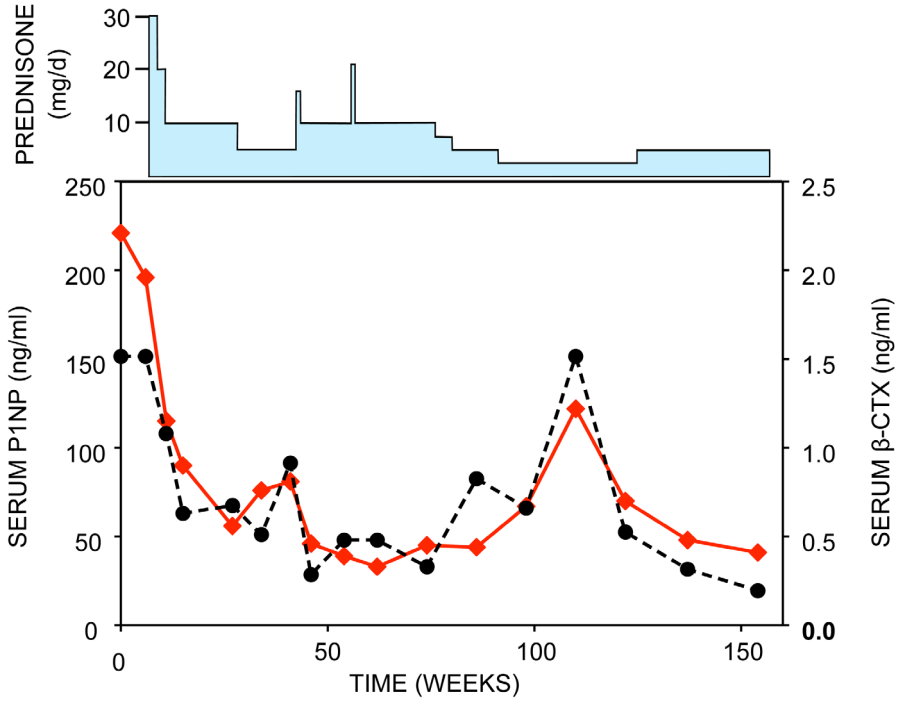


Figure 3. Biochemical markers of bone formation and resorption before and during treatment with prednisone. P1NP: diamonds and solid line; β-CTX: closed circles and interrupted line.

Table 1: Bone mineral density measurments before and after 2 years of prednisone treatment

Date	Height (cm)	BMD Spine (g/cm^2)	Change (%)	BMD Total hip (g/cm^2)	Change (%)
2-2001	193.9	1.634	-	1.410	-
4-2003	197.0	1.787	9.4	1.741	23.5
1-2005	197.0	1.855	3.8	1.820	4.5
2-2007	197.0	1.934	4.3	1.888	3.7
6-2009*	197.0	1.921	-0.7	1.895	0.4

* after 2 years of prednison. BMD: bone mineral density

Discussion

This case illustrates the beneficial effect of prednisone treatment on bone metabolism in a patient with van Buchem disease and life-threatening increased intracranial pressure. Treatment resulted in a histologically documented dramatic decrease in bone formation. Following therapy there was also no further increase in BMD at the spine and the hip. Although clinical manifestations of increased intracranial pressure improved significantly, this cannot be solely attributed to treatment with prednisone, as the patient had a ventriculo-peritoneal drain simultaneously implanted at the time of starting prednisone.

Before prednisone treatment, the patient had an increased rate of bone turnover, as assessed biochemically, associated with a continuous increase in BMD of the spine and at the hip. The biochemical markers of bone formation P1NP and osteocalcin have been previously reported to be elevated in 6 patients with van Buchem disease compared to their levels in disease carriers, being above the upper limit of the normal range in 3 of them.(15) Urinary NTX levels were higher in 4 patients with the disease compared to carriers. Bone density measured in the phalanges by radiographic absorptiometry was elevated in all these patients.(15) There are however to date no longitudinal data reported in patients with van Buchem disease. In our patient,

at least up to the age of 23 years, both biochemical markers of resorption and formation were increased. The clinical progression of the disease, which was due to bone overgrowth, as also evidenced by the steady increase in BMD, prompted us to use glucocorticoids in an attempt to arrest the process of bone accumulation.

The beneficial use of glucocorticoids has been previously reported in a patient with craniotubular hyperostosis due to an unidentified genetic defect. (16) In this patient, prednisone given for three courses of 10 weeks each, reduced serum osteocalcin but had no effect on urinary deoxypyridinoline and there were no reported changes in BMD. In a few cases with progressive diaphyseal dysplasia, a craniotubular hyperostotic disorder distinct from van Buchem disease, which is due to mutations of the gene encoding TGFβ, prednisone treatment during childhood and adolescence led to clinical(17,18) and in one case radiological improvement.(19)

Glucocorticoids have a deleterious effect on the skeleton, increasing bone fragility by systemic and local actions.(11) Their main action on bone metabolism is to decrease bone formation by inhibiting of the proliferation and differentiation of osteoblasts and increasing their rate of apoptosis.(12,20) Glucocorticoids have also been reported to increase bone resorption, particularly during the early phase of treatment, by stimulating osteoclastic activity and survival through an effect on the RANKL/OPG signaling pathway.(21-23) Consistent with these findings, studies in animals(24) and in humans(25-33) have shown that administration of glucocorticoids significantly reduce biochemical markers of bone formation, but have no effect or even increase those of bone resorption. Remarkably, administration of prednisone to our patient did not only decrease bone formation, but also bone resorption within 4 weeks of starting of treatment. Serum P1NP and β-CTX decreased and increased concurrently during alterations of prednisone dose, suggesting a tight coupling of bone resorption and formation during treatment. This was further supported by the strong correlation between the two biochemical markers of bone turnover before and during prednisone treatment.

The reason for this unique response of bone resorption to prednisone is not apparently clear but may well be related to the genetic defect of our patient with van Buchem disease. Recent studies have indicated that at least some of the negative effects of glucocorticoids on osteoblast function are due to inhibition of the canonical Wnt

signaling pathway through stimulation of the Wnt antagonists Dkk1, Sfr1 and sclerostin and activation of GSK3β.(34-36) In addition, it has been reported that in osteoblasts Wnt signalling decreases bone resorption by downregulating the expression of RANKL and upregulating that of OPG,(37-40) an action that can be reversed by glucocorticoids.(21-23) It may thus be that sclerostin is not required for the action of glucocorticoids on bone formation, as suggested by the clear reduction of bone formation in our patient in the absence of sclerostin. In contrast, sclerostin may well be important for the action of glucocorticoids on bone resorption. We propose that in the absence of sclerostin glucocorticoids may lose their ability to stimulate RANKL and decrease OPG by a mechanism that is as yet to be explored. In support of this hypothesis are the data in above mentioned patient with craniotubular hyperostosis treated with prednisone.(16) This patient was phenotypically very similar to ours and had increased bone turnover before treatment, although genetic analysis excluded abnormalities in the SOST gene. In this patient, prednisone treatment was associated with a significant decrease in serum osteocalcin but with no parallel change in urinary DPD excretion, a response compatible with that reported in other human studies. It may be therefore that sclerostin besides its critical role in the regulation of bone formation is also involved in the regulation of bone resorption as has also been reported for Dkk1, another inhibitor of the Wnt signaling pathway.(41)

The long-term follow-up of this patient with life-threatening complications as a result of excess bone formation, illustrates the beneficial effect of prednisone treatment on bone metabolism and suggests that using glucocorticoids may represent an attractive medical alternative to the currently used technically difficult and complication associated surgical treatments of such patients. The results suggest further that sclerostin may be involved in the regulation of bone resorption by a mechanism that needs to be further explored.

Acknowledgments

Special thanks to dr Pascale Chavassieux for performing the histomorphometric analysis of the bone biopsy.

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Chapter 6

Circulating sclerostin levels are decreased in patients with endogenous hypercortisolism and increase after treatment

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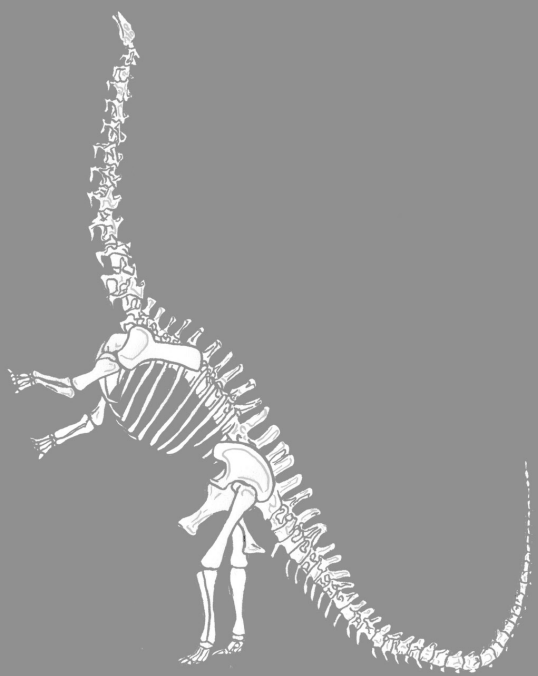

J Clin Endocrinol Metab. 2012 Oct;97(10):E1953-7.

Abstract

Context: Increased bone fragility is a frequent complication of hypercortisolism due predominantly to suppression of bone formation. Sclerostin is an osteocyte-produced negative regulator of bone formation, which is up-regulated by glucocorticoids in mice.

Objective: To assess the effect of endogenous hypercortisolism on circulating sclerostin and bone turnover in humans.

Design: We measured sclerostin, CTX, P1NP and FGF23 in blood samples of 21 patients with endogenous hypercortisolism and 21 age- and gender-matched controls. In 12 patients measurements were repeated at various time intervals after successful surgical treatment (transsphenoidal surgery or adrenalectomy).

Results: Plasma sclerostin levels were significantly decreased in patients compared to controls (112 ± 49 vs 207 ± 48 pg/ml, $p<0.001$). In the 12 patients who were evaluated after surgical treatment, sclerostin levels increased from 121.4 ± 46.5 pg/ml to 175.8 ± 78.5 pg/ml ($p=0.003$). These changes in plasma sclerostin levels were accompanied by significant increases in levels of FGF23 (from 44.2 ± 12.2 pg/ml to 84.0 ± 58.8 pg/ml , $p=0.017$) and of the bone turnover markers P1NP (from 31.7 ±18.2 ng/ml to 94.2 ± 92.2 ng/ml, $p=0.037$) and CTX (from 134.2 ± 44 pg/ml to 409.2 ± 285 pg/ml, $p=0.005$).

Conclusions: Contrary to the findings in mice, circulating sclerostin is decreased in patients with chronic endogenous hypercortisolism and increases after treatment. These findings suggest that in humans chronic exposure to glucocorticoids affects the number or function of osteocytes rather than the production of sclerostin.

Introduction

Glucocorticoid excess increases bone loss and fragility leading to glucocorticoid-induced osteoporosis (1;2). The action of glucocorticoids on bone is complex and involves local and systemic factors which adversely affect bone mass and quality. Of these, suppression of bone formation and increased rate of apoptosis of osteoblasts and osteocytes are considered major determinants of the deleterious effect of glucocorticoids on bone

In osteoblasts, glucocorticoids inhibit Wnt-signaling (3,4), a pathway essential for osteoblast proliferation, differentiation and survival, enhance the expression of RANKL and reduce that of OPG promoting also osteoclast activity (5,6). In recent years the osteocyte-derived protein sclerostin has emerged as a key inhibitor of the Wnt signaling pathway in osteoblasts. In rodents, glucocorticoids stimulated the expression of *SOST,* the gene encoding sclerostin (3) and a neutralizing antibody against sclerostin prevented glucocorticoid-induced bone loss (7). These findings suggest that glucocorticoids may exert their action on bone formation by stimulating the production of sclerostin. In the present study we tested this hypothesis in humans. For this, we studied patients with endogenous hypersecretion of cortisol because in glucocorticoid-treated patients disease-related factors may have independent effects on bone metabolism and fragility and may also affect the synthesis of sclerostin(8,9).

Patients and Methods

Patients

We studied 21 consecutive patients with endogenous hypercortisolism. The diagnosis and causes of hypercortisolism were established as previously described (10). Fifteen patients had ACTH producing pituitary adenomas, 2 had adrenal adenomas, 4 had ectopic ACTH production (unknown origin 2, neuroendocrine endocrine tumor 1 and metastatic melanoma 1). Non-fasting blood samples were obtained from all patients at different times of the day, mainly in the afternoon.

In 12 of the 21 patients blood samples were also obtained at various time intervals following surgical treatment and achievement of biochemical remission. Eight of these patients were treated by transphenoidal surgery and 4 by bilateral adrenalectomy. Remission was established by an adequate suppression of plasma cortisol after 1

mg dexamethasone overnight and disappearance of clinical signs and symptoms of hypercortisolism postoperatively (10). At the time of blood collection 8 of the 12 patients were receiving substitution therapy with hydrocortisone (n=7) or dexamethasone (n=1) and 2 patients used L-thyroxine for secondary hypothyroidism.

Preoperative levels of sclerostin and bone turnover markers were compared to those of 21 age- and gender-matched healthy volunteers who were recruited by advertisements. None of these individuals used any medication or had any illness requiring medical attention. Similar to patients, non-fasting blood samples were obtained from controls in the afternoon.
All studied subjects (healthy and patients) had normal renal function and serum calcium and phosphate concentrations.

Biochemical measurements
Creatinine was measured by semi-automated techniques. Intact PTH was measured by the Immulite 2500 (Siemens diagnostics, Breda, Holland). Aminoterminal propeptide of type 1 procollagen (P1NP) and beta C-terminal telopeptide (CTX) were determined by the E-170 system (Roche BV, Woerden, the Netherlands). Sclerostin was measured by an electrochemiluminescence assay (MSD 96-well MULTI-ARRAY Human Sclerostin Assay, Gaithersburg, MD, USA), as previously described (11). With this assay no sclerostin could be detected in the serum of 19 patients with sclerosteosis, whereas sclerostin was detectable in serum of 77 healthy individuals. In previous studies we measured sclerostin in serum while in the present study we used EDTA plasma samples due to their availability. Because of earlier reported differences in sclerostin levels in plasma and serum (12), we first measured sclerostin levels in simultaneously obtained serum and plasma samples from 26 individuals with serum sclerostin levels ranging from 16.2 to 94.7 pg/ml. Compared to serum, sclerostin levels were on average 3.6-fold higher in plasma but the two values were highly and significantly correlated (r=0.91, p=0.001).
Fibroblast Growth Factor 23 (FGF23) levels, as an independent index of osteocyte function, were measured with the Intact FGF23 ELISA kit (Kainos laboratories, Tokyo, Japan). The intra- and inter–assay coefficients of variation were 9 % and 11% respectively.

Statistical analysis

Values are reported as mean ± standard deviation, unless otherwise stated. Differences between patients and controls were assessed by t-test. Of the 12 patients who were studied during biochemical remission, differences in sclerostin levels and other biochemical markers before and after surgical treatment were analyzed by paired t-tests. Correlations between markers and changes in markers were assessed by Pearson's correlation. Normality of distribution was assessed by Shapiro-Wilk tests. Levels of CTX and FGF23 were log transformed because of skewness. Data were analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). A p-vale of <0.05 was considered significant.

Results

Baseline biochemistry

Group characteristics and relevant biochemical parameters at baseline in patients and controls are shown in table 1. Of the 21 patients with endogenous hypercortisolism included in the study, 16 were female (11 premenopausal and 5 postmenopausal) and 5 were male. Mean age was 41.3 ± 12.7 years, mean weight 86.6 ± 17.1 kg, and mean BMI 31.2 ± 6.6 kg/m^2.

Mean plasma sclerostin levels were significantly lower in patients compared to controls (112.0 ± 49.0 pg/ml vs 207.2 ± 48.4 pg/ml, $p<0.001$), while there were no significant differences between the two groups in levels of P1NP, CTX or FGF23 (Table 1).

Mean afternoon cortisol concentrations of patients was 0.63 ± 0.31 μmol/l and there was no relationship between plasma cortisol and sclerostin levels($r=-0.28$, $p=0.31$), or P1NP ($r=0.45$, $p=0.09$); however, plasma cortisol concentrations were significantly correlated with serum CTX levels ($r=0.65$, $p=0.009$). Serum levels of CTX and P1NP were also significantly correlated in these patients ($r=0.55$, $p=0.03$).

Changes in biochemical parameters after treatment

Mean plasma cortisol concentration was 0.18 ± 0.18 μmol/l in the 12 patients who were followed after surgical treatment. Changes of all studied biochemical parameters during remission of the disease are shown in Figure 1. Remission was associated with a significant increase in mean sclerostin levels (from 121.4 ± 46.5 pg/ml to 175.8 ±78.5 pg/ml; $p=0.003$). Similarly, P1NP levels increased from 31.7 ±18.2 ng/

ml to 94.2 ± 92.2 ng/ml (p=0.037), and CTX levels increased from 134.2 ± 44 pg/ml to 409.2 ± 285 pg/ml (p=0.005). Plasma FGF23 levels also increased during biochemical remission from 44.2 ± 12.2 to 84.0 ± 58.8 (p=0.017). Serum creatinine and plasma PTH concentrations, which may affect circulation sclerostin did not change significantly (68.8 ± 14.7 μmol/l to 71.8 ± 14.1 μmol/l, p=0.59 and 4.9 ± 2.3 pmol/l to 4.3 ± 2.1 pmol/l, p=0.60, respectively).

The median time of blood sampling after surgical treatment was 3 months (range 1 week to 18 months). There was a positive correlation between the percent changes in plasma sclerostin levels and time of surgery (r=0.68, p=0.015). There was no correlation between sclerostin levels and changes of bone turnover markers.

Table 1. mean age and biochemical parameters of patients with endogenous hypercortisolism and age-and-gender matched healthy controls

	controls	patients	p-value
male: female	5: 16	5: 16	-
Age (years)	41.6 ± 11.8	41.3 ± 12.7	0.66
P1NP (ng/ml)	44.9 ±14.3	36.9 ± 21.9	0.12
CTX (pg/ml)	224 ± 144	229 ± 224	0.94
Sclerostin (pg/ml)	207 ± 48	112 ± 49	<0.001
FGF23 (pg/ml)	37.5 ± 16.9	42.8 ± 12.1	0.25
Creatinine (μmol/l)	68.6 ± 12.8	66.9 ± 13.8	0.62
PTH (pmol/l)	-	6.6 ± 3.1	-
Calcium (mmol/l)	2.25 ± 0.07	-	-
Phosphate (mmol/l)	1.28 ± 0.14	-	-

Discussion

Since its discovery a decade ago, sclerostin has emerged as a key negative regulator of bone formation and glucocorticoids were reported to stimulate the expression of the *SOST* gene, which encodes sclerostin (3). Contrary to this finding, we show here that plasma sclerostin levels are significantly decreased in patients with endogenous hypercortisolism and increase after surgical treatment and achievement of clinical and biochemical remission.

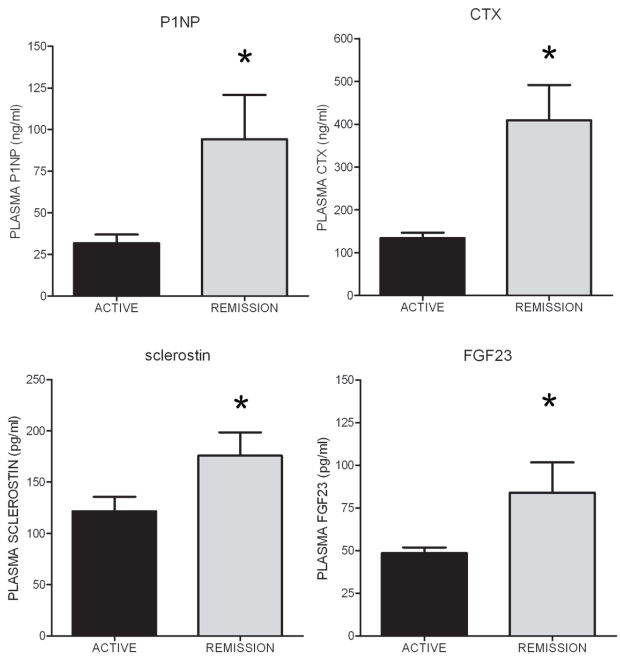

Figure 1. Mean (SD) levels of biochemical markers of bone turnover, sclerostin and FGF23 in patients with endogenous hypercortisolism at baseline (black bars) and during biochemical remission (grey bars). *$p<0.05$

In bone, sclerostin is exclusively produced by osteocytes (13), cells that are directly affected by glucocorticoids which promote their apoptosis (1). Recent evidence suggests that glucocorticoids may also induce autophagy in osteocytes (14), and/or may increase oxidative stress (15). We, therefore, propose that the decreased sclerostin levels of patients with hypercortisolism are due to decreases in osteocytes function and/or number rather than to a direct, inhibitory, effect of glucocorticoids on sclerostin production. The increases in sclerostin levels during biochemical remission may be due either to the recovery of the function of the osteocytes, following the cessation of the metabolic stress, or to an increase in the number of osteocytes following the removal of the apoptotic effect of glucocorticoids

To further explore this notion, we measured circulating FGF23 in our patients before and after treatment. FGF23 is also produced by osteocytes but its production is not affected by glucocorticoids (3). It can, therefore, be considered as an independent parameter of the function and/or number of osteocytes. The observed, significant

increases in the levels of FGF23 after treatment strongly support the hypothesis that the increases in sclerostin levels represent a restoration of osteocyte numbers and/or function. Consistent with this hypothesis is also the observed increase in the biochemical markers of bone turnover. Improvement in osteocyte number and/or function may be responsible for the reversibility of the fracture risk following discontinuation of glucocorticoid therapy (16).

Bone loss following the exogenous administration of glucocorticoids is biphasic with an initial rapid phase occurring during the first months of treatment followed by a prolonged phase of much slower bone loss. It has been postulated that during the initial phase an imbalance between bone resorption and bone formation, in favor of resorption, is the predominant pathogenetic factor for bone loss while during the second, chronic, phase reduced bone formation and osteocyte apoptosis are the main determinants of bone strength (17). Our findings in patients with chronic endogenous hypercortisolism are consistent with this sequence of events. Thus, in the presence of chronic glucocorticoid excess sclerostin does not seem to affect bone formation but its values in blood may be a measure of the function and/or number of osteocytes. However, sclerostin may very well be directly involved in the initial phase of bone loss induced by glucocorticoids. This could not be studied in our patients but we previously showed that treatment of a patient with sclerostin deficiency with prednisone reduced both resorption and formation markers and we suggested that sclerostin plays an important role in the modulation of bone resorption by glucocorticoids while it does not affect their action on bone formation (18). Furthermore, mice treated with dexamethasone were protected from glucocorticoid-induced bone loss, when they were simultaneously treated with a neutralizing antibody against sclerostin (7). In these mice the protective effect of the sclerostin antibody was mainly due to the stabilization of bone resorption, with no evident effect on bone formation. Sclerostin is thought to act predominantly as an inhibitor of bone formation but a recent study has shown that sclerostin can also stimulate osteoclast differentiation and function, by a RANKL-mediated mechanism in osteocytes (19). In addition, inhibition of RANKL by OPG reduces the rate of osteocyte apoptosis in glucocorticoid-treated mice (20). Thus, although we found no evidence for a role of sclerostin in glucocorticoid-induced suppression of bone formation during long-standing endogenous hypercortisolism, sclerostin may play a role in the early phase of glucocorticoid-induced bone loss. Further studies are needed to address these questions.

In conclusion, in this study we found that patients with endogenous hypercortisolism have decreased circulating sclerostin levels, which increase during biochemical remission of the disease and we propose that these changes in sclerostin levels are due to the detrimental effect of glucocorticoids on osteocytes rather than to a direct effect of glucocorticoids on sclerostin synthesis.

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Chapter 7

Patients with primary hyperparathyroidism have lower circulating sclerostin levels than euparathyroid controls

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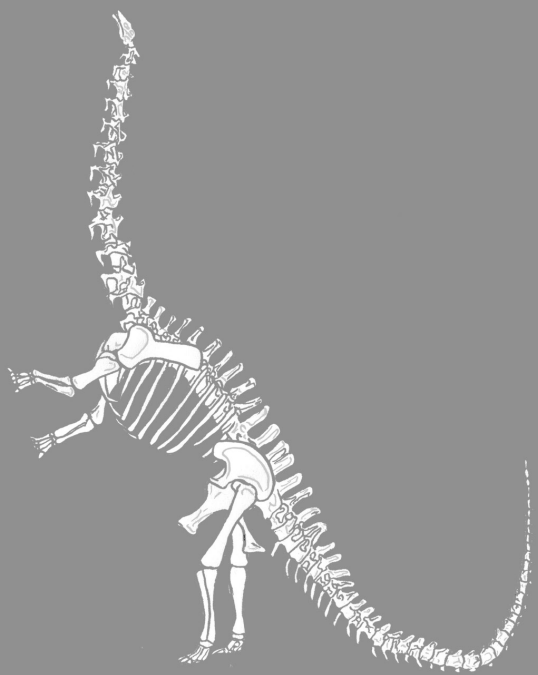

Eur J Endocrinol. 2010 Nov;163(5):833-7

Abstract

Objective

In vitro and in vivo studies in animal models have shown that parathyroid hormone (PTH) inhibits the expression of the *Sost* gene which encodes sclerostin, an osteocyte-derived negative regulator of bone formation. We tested the hypothesis that chronic PTH excess decreases circulating sclerostin in humans.

Design

We studied 25 patients with elevated serum PTH concentrations due to primary hyperparathyroidism (PHPT) and 49 patients cured from PHPT after successful parathyroidectomy (EuPTH).

Methods

We measured plasma PTH and serum sclerostin levels and the serum markers of bone turnover alkaline phosphatase, P1NP, and β-CTX.

Results

As expected by the design of the study, mean plasma PTH was significantly higher ($p < 0.001$) in PHPT patients (15.3 pmol/l; 95%CI: 11.1-19.5) compared to that of EuPTH controls (4.1 pmol/l; 95%CI: 3.6- 4.5). PHPT patients had significantly lower serum sclerostin values compared to EuPTH subjects (30.5 pg/ml; 95%CI: 26.0-35.1 vs 45.4 pg/ml; 95%CI: 40.5-50.2; $p<0.001$) and to healthy controls (40.0 pg/ml; 95%CI: 37.1-42.9; $p=0.01$). Plasma PTH concentrations were negatively correlated with serum sclerostin values ($r= -0.44$; $p<0.001$). Bone turnover markers were significantly correlated with PTH, but not with sclerostin.

Conclusion

Patients with primary hyperparathyroidism have significantly lower serum sclerostin values compared to PTx controls with normal PTH concentrations. The negative correlation between PTH and sclerostin strongly suggests that *SOST* is downregulated by PTH in humans.

Introduction

Parathyroid hormone (PTH) exerts its calciotropic action by acting directly on bone and kidney and indirectly on the intestine to increase the transport of calcium to the circulation. The skeletal effect of PTH is to increase the rate of remodeling (1), and it is generally believed that this effect is achieved by the binding of PTH to its specific receptor (PTHR1) on stromal/osteoblastic cells of the bone marrow. This in turn stimulates the production of RANK-ligand (RANKL) and decreases that of its decoy receptor osteoprotegerin (OPG) (2-5). Recent in vitro and animal studies suggest, however, that at least some of the effects of PTH on bone are also exerted by specific binding of the hormone to PTHR1 in osteocytes, resulting in inhibition of the expression of the *Sost* gene (6-9). This gene encodes sclerostin, a protein exclusively expressed in osteocytes in the skeleton (10), which decreases bone formation by binding to LRP5/6, resulting in inhibition of the Wnt signaling pathway in osteoblasts (11, 12).

Whether chronic PTH excess has similar effects on sclerostin secretion in humans as in animal models has not so far been investigated. In the present study, we tested the hypothesis that chronic hypersecretion of PTH, as seen in patients with hyperparathyroidism, may decrease sclerostin secretion, and that PTH may thus represent a potential regulator of sclerostin production in humans. To this effect, we measured sclerostin in serum of patients with untreated primary hyperparathyroidism (PHPT) and in a control group of patients with PHPT after establishment of cure following parathyroidectomy (PTx).

Subjects and Methods

a. Patients

Thirty-four consecutive patients with primary hyperparathyroidism (PHPT), which was untreated, persistent, or recurrent following parathyroidectomy (PTx), and 54 patients cured after successful PTx (EuPTH) were studied. Inclusion criteria included willingness to participate in the study, no impairment in renal function (serum creatinine levels <120 µmol/l), adequate vitamine D status (25-hydroxy vitamin D levels >50nmol/l) and no use of bone and mineral metabolism modifying agents such as bisphosphonates, calcimimetics or glucocorticoids.

We defined PHPT as plasma PTH concentrations above the upper limit of the normal laboratory reference range (>8pmol/l) in the presence of increased serum calcium concentrations (>2.55 mmol/l).
Patients with EuPTH were included when cure was confirmed by post-operative normalization of serum PTH and calcium concentrations, which was sustained for at least 6 months after PTx.
As per inclusion criteria, 9 patients were excluded from the PHPT group, 3 because of impaired renal function and 6 because of use of bisphosphonates, and 6 were excluded from the EuPTH group because of use of bisphosphonates.

The study was approved by the Medical Ethics Committee of the Leiden University Medical Center, and informed consent was obtained from all patients.

b. Methods.

Serum biochemistry

Serum calcium adjusted for albumin binding, phosphate, and creatinine were measured by semi-automated techniques. Serum alkaline phosphatase activity (ALP) was measured using a fully automated P800 modulator system (Roche BV, Woerden, Holland). P1NP (a marker of bone formation) and β-CTX (a marker of bone resorption) were determined using the E-170 system (Roche BV, Woerden, Holland). Plasma PTH was measured using the Immulite 2500 (Siemens diagnostics, Breda, Holland) and serum 25-hydroxyvitamin D (25-OHD) was measured using the LIAISON® 25-OH Vitamin D TOTAL assay (DiaSorin S.A./N.V., Bruxelles, Belgium)

Sclerostin measurement

Sclerostin was measured in serum by an electrochemiluminescence assay (MSD® 96-well MULTI-ARRAY® Human Sclerostin Assay, Gaithersburg, Maryland, USA) which uses two polyclonal antibodies raised against the whole sclerostin molecule. The sclerostin standard for the assay is produced in a NS0 derived myeloma cell line, and the purity is checked by SDS-PAGE gel with silver stain. In our hands, the precision and reproducibility of the assay were $< 6\%$ and $< 15\%$, respectively, the detection limit was ± 1 pg/ml, and the detection range was 1 to 10,000 pg/ml.
Sclerostin was measured in serum of 77 healthy subjects (30 male and 47 female,

aged 20 to 77 years). All had normal serum calcium concentrations, renal function and bone turnover and none were using bisphosphonates, the calcimimetic cinacalcet or glucocorticoids. Sclerostin was detected in serum of all healthy subjects; mean 40.0 pg/ml (95%CI = 37.1 - 42.9 pg/ml), range 12.4 to 68.19 pg/ml, while it was undetectable in serum of 3 patients with sclerosteosis .

c. Statistical analysis

Data was analysed using SPSS 16.0 (SPSS Inc. Chicago, USA). Between groups differences in baseline characteristics and serum biochemistry were assessed by student's t-test. Pearson correlation coefficients were calculated to assess correlations between PTH (after logarithmic transformation), sclerostin and biochemical markers of bone turnover. A probability level of random difference of 0.05 was considered significant.

Results

Baseline characteristics

There were no differences in age, gender, weight or BMI between patients with PHPT and EuPTH controls (table 1).

As expected by inclusion criteria, mean serum calcium and PTH concentrations were significantly higher and those of phosphate significantly lower in the PHPT group compared to the EuPTH group. There were no differences in serum 25-OHD or creatinine concentrations between the two groups.

Table 1. Subject characteristics.

	PHPT	EuPTH	p-value*
Male : Female	10:15	13:36	0.41
Age (years)	59.6 ± 16.7	62.4 ± 10.9	0.44
Weight (kg)	80.0 ± 18.2	81.1 ± 15.3	0.78
BMI (kg/m^2)	27.0 ± 6.0	28.1 ± 4.6	0.42

Values are given as mean ± standard deviation. BMI = body mass index. * PHPT vs EuPTH

Patients with PHPT had significantly higher levels of biochemical markers of bone formation (P1NP) and bone resorption (β-CTX) compared to EuPTH controls. Combining all patients, there was a significant positive correlation between plasma PTH concentrations and the concentrations of all three measured biochemical markers of bone turnover (ALP: r=0.23, p=0.047; P1NP: r=0.45, p<0.001; β-CTX: r=0.54, p<0.001). There was also a significant correlation between PTH and P1NP (r=0.51, p=0.009) in the PHPT group, but not between PTH and ALP (r=0.35, p=0.085), or PTH and β-CTX (r=0.31, p=0.13). In the EuPTH group alone PTH was not correlated with any of the biochemical markers of bone turnover (Tabel 2).

Serum sclerostin

Mean serum sclerostin level of patients with PHPT (30.5 pg/ml, 95%CI: 26.0-35.1) was significantly lower than that of patients with EuPTH and healthy controls (45.4 pg/ml, 95%CI: 40.5-50.2; p<0.001, and 40.0 pg/ml, 95%CI: 37.1-42.9; p=0.01, respectively) (Figure 1). There was no significant difference in mean sclerostin values between EuPTH and healthy subjects (p=0.13).

There was no significant correlation between PTH and sclerostin concentrations within each individual group of patients but there was a significant negative correlation between sclerostin and PTH when all patients were pooled together (r=-0.44, p<0.001) (Figure 2).

There was no significant relationship between serum sclerostin and biochemical markers of bone turnover in patients with PHPT or in all patients combined.

Table 2. Biochemical measurements

	PHPT	EuPTH	Reference range	p-value*
Calcium homeostasis				
PTH (pmol/l)	15.3 ± 10.7	4.1 ± 1.6	1.5 - 8.0	<0.001
Calcium (mmol/l)	2.61 ± 0.13	2.26 ± 0.12	2.15 - 2.55	<0.001
Phosphate (mmol/l)	0.92 ± 0.14	1.14 ± 0.24	0.90 - 1.50	<0.001
25 (OH) D (nmol/l)	53.1 ± 34.3	53.9 ± 20.2	30 -120	0.90
Creatinine (µmol/l)	80.2 ± 18.1	76.8 ± 14.7	44 - 80	0.39
Bone turnover				
ALP (U/l)	87.0 ± 23.0	75.7 ± 23.6	40 - 120	0.055
P1NP (ng/ml)	45.9 ± 16.9	34.0 ± 15.5	16 - 80	0.004
β-CTX (ng/ml)	0.32 ± 0.15	0.17 ± 0.11	0.01– 0.66	<0.001

Values are given as mean ± standard deviation.

PHPT = primary hyperparathyroidism; EuPTH= euparathyroid controls.

* PHPT vs EuPTH

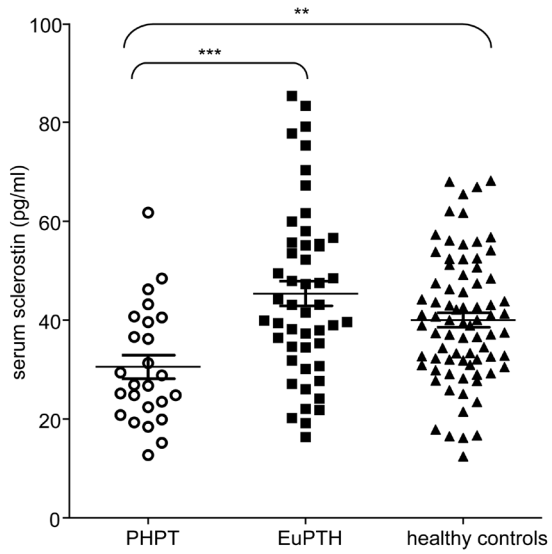

Figure 1. Serum sclerostin levels in PHPT, EuPTH and healthy subjects.
PHPT = primary hyperparathyroidism; EuPTH= euparathyroid controls.
*** p <0.001 ** p =0.01 (student's t-test).

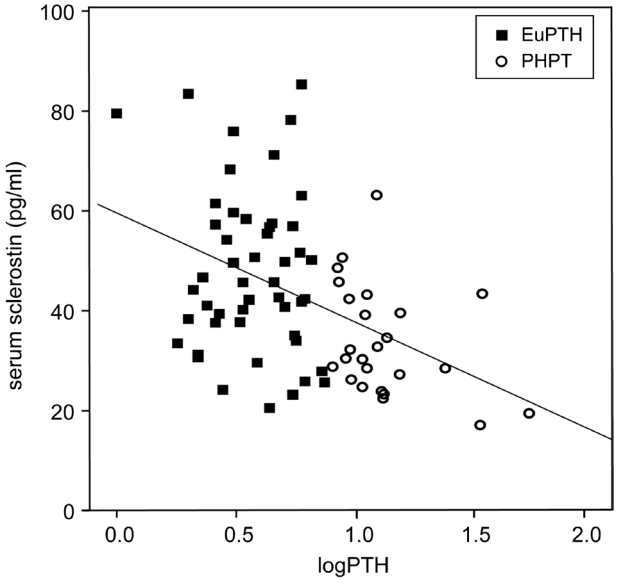

Figure 2. Relationship between circulating sclerostin and PTH levels.
r=-0.44 , p<0.001 (pearson's correlation). PTH levels were log transformed because of skewness.
PHPT = primary hyperparathyroidism; EuPTH = euparathyroid controls.

Discussion

Data from our study demonstrate that in humans, chronic PTH excess as observed in patients with primary hyperparathyroidism, is associated with a significant decrease in circulating sclerostin and that there is a significant negative correlation between PTH and serum sclerostin levels. Taken together these results suggest that, similar to animal models (6-9), PTH has a regulatory role on sclerostin production also in humans.

Sclerostin, a glycoprotein expressed by osteocytes in bone and encoded by the *SOST* gene, has emerged in recent years as an important regulator of bone formation in humans as well as in animals (13, 14). Inactivating mutations of the *SOST* gene leading to sclerostin deficiency have been shown to be associated with the rare skeletal disorder sclerosteosis, which is characterized by a marked increase in bone mass (13). Deletion of the *Sost* gene in mice have also been shown to increase bone formation, bone mass and bone strength (14). Moreover, inhibition of sclerostin secretion by a monoclonal antibody to sclerostin has been shown to increase bone formation and bone mass in rodents, primates and humans (14-16). Conversely, transgenic mice overexpressing *Sost* have low bone mass and impaired biomechanical competence (17). The mechanism of action of sclerostin to decrease bone formation involves inhibition of the Wnt signaling pathway (11, 12), although its precise molecular mechanism and factors controlling its secretion are as yet to be determined. Recent animal studies have shown that mechanical loading and high PTH levels downregulate the expression of *Sost* in osteocytes and decrease the production of sclerostin resulting in stimulation of bone formation (8, 18).

Human studies of sclerostin regulation have lagged behind due to lack of non-invasive techniques to determine sclerostin production. A number of assays have been recently developed for the measurement of sclerostin in blood. In our study, we used a sclerostin assay which proved to have excellent performance characteristics in our hands. Sclerostin was detectable in serum in all healthy subjects studied, suggesting that the protein is secreted and enters the circulation, while it was undetectable in three patients with sclerosteosis in whom it was measured. We chose to study patients with primary hyperparathyroidism in order to mimic as closely as possible the effect of chronic PTH excess on sclerostin, as previously studied in animals (6, 8). In addition, we chose to use as controls patients with primary hyperparathyroidism

cured after parathyroidectomy to exclude potential confounding factors, other than PTH excess.

We show here that serum sclerostin levels are significantly decreased in patients with chronic PTH excess due to PHPT compared to EuPTH and healthy subjects. PTH has been shown to decrease *SOST* transcription in vitro (6, 7), and continous and intermitent chronic administration of PTH to rodents is associated with decreased *Sost* mRNA and sclerostin expression in osteocytes (6, 7, 9). Moreover, transgenic mice expressing a constitutively active PTH receptor in osteocytes exhibit decreased expression of sclerostin and increased Wnt signaling associated with increased bone mass (8). Additional evidence for an interaction between PTH and *Sost*/sclerostin was recently provided by a study showing that the anabolic actions of PTH on bone was blunted in *Sost*-overexpressing mice (19). In keeping with the notion of a regulatory role of PTH for sclerostin production, our data show a significant correlation between circulating PTH and sclerostin. These data extend those of Mirza et al (20) who recently reported a negative relationship between serum PTH and sclerostin in healthy postmenopausal women, and those of Drake et al (21), who showed that intermittent PTH treatment decreased serum sclerostin levels in postmenopausal women.
As expected in the presence of chronic PTH excess, patients with PHPT had increased bone turnover as indicated by increased biochemical parameters of bone formation and resorption. There was a significant relationship between circulating PTH concentrations and serum P1NP and β-CTX. We did not however, find a significant relationship between biochemical parameters of bone turnover and serum sclerostin, either in patients with PHPT or the combined group of PHPT and EuPTH subjects. This lack of correlation between sclerostin and bone turnover markers was previously reported in healthy postmenopausal women (20).

The actions of PTH on bone are complex and involve a variety of signaling pathways in bone marrow stroma cells, osteoblasts and osteocytes (22, 23). Despite the significant progress in our understanding of the actions of PTH on bone it should be appreciated that the cellular and molecular actions of PTH, which determine the action of the hormone on bone remodeling and bone balance have only been partially unraveled and studies are needed to further elucidate these actions.

Acknowledgments

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Chapter 8

Distinct effects of pioglitazone and metformin on circulating sclerostin and biochemical markers of bone turnover in men with type 2 diabetes mellitus

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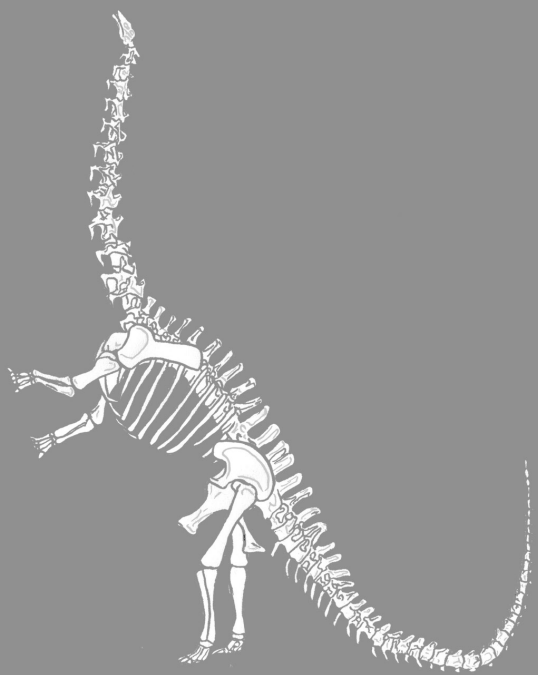

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Abstract

Objective

Patients with Type 2 Diabetes Mellitus (T2DM) have an increased risk of fractures and thiazolidinediones (TZDs) increase this risk. TZDs stimulate the expression of sclerostin, a negative regulator of bone formation, in vitro. Abnormal sclerostin production may, therefore, be involved in the pathogenesis of increased bone fragility in patients with T2DM treated with TZDs.

Methods

We measured serum sclerostin, P1NP and CTX in 71 men with T2DM treated with either pioglitazone (PIO) 30 mg once daily, or metformin (MET) 1000mg twice daily. Baseline values of sclerostin and P1NP were compared to those of 30 healthy male controls

Results

Compared to healthy controls, patients with T2DM had significantly higher serum sclerostin levels (59.9 pg/ml vs 45.2 pg/ml, $p<0.001$), but similar serum P1NP levels (33.6 ng/ml vs 36.0 ng/ml, $p=0.39$). After 24 weeks treatment, serum sclerostin levels increased by 11% in PIO-treated patients, and decreased by 1.8% in MET-treated patients ($p=0.018$). Changes in serum sclerostin were significantly correlated with changes in serum CTX in all patients ($r=0.36$, $p=0.002$), and in PIO-treated patients ($r=0.39$, $p=0.020$), but not in MET-treated patients ($r=0.17$, $p=0.31$).

Conclusions

Men with T2DM have higher serum sclerostin levels than healthy controls and these levels further increase after treatment with PIO, which is also associated with increased serum CTX. These findings suggest that increased sclerostin production may be involved in the pathogenesis of increased skeletal fragility in patients with T2DM in general, and may specifically contribute to the detrimental effect of TZDs on bone.

Introduction

Patients with type 2 diabetes mellitus (T2DM) have an increased risk of fractures (1) but the mechanism(s) responsible for the increased bone fragility remain unclear. Moreover, blood glucose lowering therapy using thiazolidinediones (TZDs), has also been reported to cause bone loss (2) and to further increase the risk of fractures (3, 4). Activation of PPAR-γ and preferential stimulation of the differentiation of bone marrow mesenchymal stem cells into adipocytes at the expense of osteoblasts has been proposed as potential mechanism for these effects of TZDs on bone (5). TZDs have also been recently reported to stimulate sclerostin synthesis in vitro, suggesting an additional mechanism for the detrimental effects of TZDs on bone (6). Sclerostin is a glycoprotein synthesized in bone by osteocytes which reduces bone formation (7, 8) and serum sclerostin levels have been found to be significantly correlated with fracture risk in postmenopausal women (9). Abnormal sclerostin production may contribute to the pathogenesis of bone fragility of patients with T2DM as well as in the actions of TZDs on bone. To test this hypothesis we measured serum sclerostin and biochemical markers of bone turnover in men with T2DM before and after treatment with the TZD pioglitazone.

Patients and Methods

We studied 71 men with T2DM who participated in the PIRAMID study (Pioglitazone Influence on Triglyceride Accumulation in the Myocardium in Diabetes). The design and results of this study (10) as well other metabolic parameters of the patients including whole body insulin sensitivity (11) have been reported previously. In brief, this was a 24-week prospective, randomized, double-blind, double-dummy with active comparator, 2-center parallel group intervention study. Men with uncomplicated T2DM, aged 45 to 65 years, were included in the study. After a 10-week wash out period of previous medications, patients were treated with glimepiride for 8 weeks and were subsequently randomized to pioglitazone (15mg once daily, titrated to 30 mg once daily after 2 weeks) or metformin (500 mg twice daily, titrated to 1000 mg twice daily) and matching placebo to be taken in addition to glimepiride throughout the study. Blood samples were taken after an overnight fast at baseline and after 24 weeks of treatment and were stored at -80 C until assayed .

Baseline data including serum sclerostin and P1NP values were compared to those of 30, previously described, healthy male volunteers (12). All of these subjects had normal serum calcium concentrations, renal function and bone turnover markers and were not using medications that could affect calcium or bone metabolism.
The study was approved by the Medical Ethics Committees of the two participating Centers and informed consent was obtained from all participants in the study.

Serum biochemistry
Sclerostin was measured by a highly sensitive electrochemiluminescence assay (Mesoscale discoveries, Gainsburg, USA) as previously described (13). The intra-precision of the assay was 6% and the inter-precision 10%. All samples from individual patients were measured in the same assay. Serum calcium adjusted for albumin binding, phosphate, and creatinine were measured by semi-automated techniques. Procollagen Type 1 Amino-terminal Propeptide (P1NP) and carboxy-terminal cross-linking telopeptide of type I collagen (CTX) were measured by the E-170 system (Roche BV, Woerden, Holland).

Statistical analysis
Data are expressed as mean ± standard deviation (unless otherwise stated). Absolute changes and percentage changes in biochemical parameters between baseline and end-of-study values were calculated for each subject. Differences between groups were assessed by student's t- tests. Differences in sclerostin levels between patients and healthy controls were corrected for BMI and weight using a linear mixed model. Within treatment group differences in percentage changes in measured parameters were tested by one-sample t-test . Correlations were assessed by Pearsons correlation tests. Statistical analysis was performed using the SPSS 17.0 software (SPSS Inc. Chicago, USA). A p-value of <0.05 was considered to be statistically significant.

Results

Baseline
Mean age of patients with T2DM (56.5 ± 5.6 years) was comparable to that of controls (55.0 ± 16.4 years, $p=0.50$), but patients were heavier (patients: 92.2 ±13.6 kg, controls: 80.7 ± 12.1 kg, $p<0.001$) and had a higher BMI (patients: 28.7 ± 3.4

kg/m2, controls: 25.4 ± 4.0 kg/m², p<0.001) (Table 1).

Patients with T2DM had significantly higher serum sclerostin levels compared to healthy controls (59.9 pg/ml; 95%CI: 55.2 - 64.8 pg/ml vs 45.2 pg/ml; 95%CI: 40.6 - 49.8 pg/ml, p<0.001) (Figure 1A). This difference in sclerostin levels remained significant after adjusting for BMI or weight (p=0.008). In patients with T2DM, serum sclerostin levels were positively correlated with serum fasting insulin levels (r=0.41, p<0.001), but not with HbA1c (r=0.07, p=0.57), weight (r=0.07, p=0.58) or BMI (r=0.18, p=0.12). There was no difference in mean serum P1NP levels between patients and controls (33.6 ng/ml; 95%CI: 31.0 - 36.6 ng/ml; and 36.0 ng/ml; 95%CI: 30.7 - 40.8 ng/ml respectively, p=0.39) (Figure 1B).

Table 1. Baseline anthropometric and biochemical data of male patients with T2DM and healthy controls. Values are given as mean ± standard deviation.

	Controls (n=30)	Patient (n=71)	p-value
Age (years)	55.0 ± 16.4	56.5 ± 5.6	0.59
Weight (kg)	80.7 ± 12.1	92.2 ± 13.6	<0.001
BMI (kg/m²)	25.4 ± 4.0	28.6 ± 3.3	<0.001
Sclerostin (pg/ml)	45.2 ± 12.8	59.2 ± 19.4	<0.001
P1NP (ng/ml)	36.0 ± 13.8	33.8 ± 12.2	0.43
CTX (pg/ml)	-	311 ± 14	
Calcium (mmol/l)	2.27 ± 0.10	2.29 ± 0.10	0.36
Phosphate (mmol/l)	1.05 ± 0.12	0.99 ± 0.17	0.41

Response to treatment

There was no significant difference in age, weight and time since diagnosis of T2DM between the two treatment groups (Table 2). Baseline values and changes of plasma glucose, insulin, and HbA1C after 24 weeks of treatment have been previously reported (10). Baseline values were similar in the two treatment groups and improved significantly, resulting in a similar degree of glycemic control during the 24 weeks of

treatment with either metformin or pioglitazone.
Baseline values of serum sclerostin and of biochemical markers of bone turnover and calcium metabolism were similar between the two treatment groups (Table 2). The two treatment regimens had, however, different effects on serum sclerostin and bone marker levels (Figure 2). Serum sclerostin levels increased by 11% (95%CI: 2.26-19.8%, p=0.019) after 24 weeks of treatment with pioglitazone, but there was no significant change in these levels in patients treated with metformin (-1.8%; 95%CI: -8.8-5.0%, p=0.42). The difference in sclerostin response between the two treatment groups was significant (p=0.018).

Serum P1NP levels increased by 3.7% (95%CI: -3.9%-11.3%, p=0.35) in the pioglitazone group, but decreased significantly by 19.1% (95%CI: -25.5%--12.8%, p<0.001) in the metformin group (between group difference p<0.001). Serum CTX levels increased by 16.8% (95%CI:4.4%-29.1%, p=0.012) after treatment with pioglitazone, but decreased by 19.0% (95%CI:-27.1%--10.9%, p<0.001) in the metformin treated group (between group difference p<0.001). There was no significant change in serum calcium, phosphate or creatinine concentrations after 24 weeks of treatment with either agent.

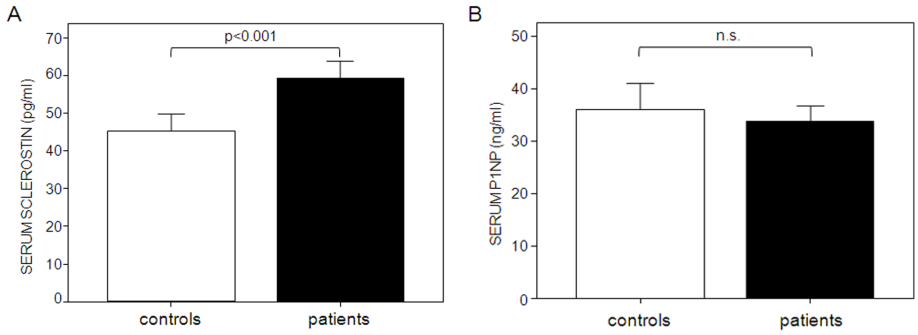

Figure 1. Serum sclerostin (A) and P1NP (B) levels in male patients with type 2 diabetes mellitus and healthy male controls. Bars represent standard error of the mean.
p-values: significance of differences between groups (student's t-tests). n.s.: non-significant

Table 2. Baseline anthropometric and biochemical data of male patients with T2DM treated with either metformin or pioglitazone. Values are given as mean ± standard deviation.

	metformin (n=37)	pioglitazone (n=34)	p-value
Age (years)	56.5 ± 5.4	56.4 ± 5.9	0.99
Weight (kg)	93.2 ± 14.6	91.1 ± 12.6	0.50
BMI (kg/m²)	29.3 ± 3.8	28.2 ± 3.0	0.17
Time since diagnosis (years)	3.6 ± 2.5	4.6 ± 2.8	0.11
Sclerostin (pg/ml)	62.4 ± 18.9	58.5 ± 22.0	0.42
P1NP (ng/ml)	35.1 ± 11.7	32.0 ± 12.7	0.28
CTX (pg/ml)	381 ± 380	294 ± 140	0.40
Calcium (mmol/l)	2.29 ± 0.10	2.28 ± 0.11	0.92
Phosphate (mmol/l)	1.00 ± 0.16	1.00 ± 0.18	0.60
Creatinine (μmol/l)	76.6 ± 17.3	77.3 ± 12.7	0.49

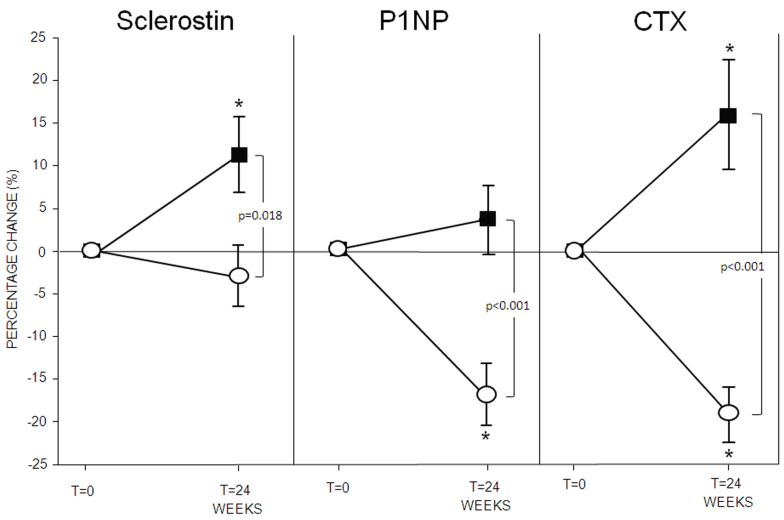

Figure 2. Mean percentage changes of serum levels of sclerostin, P1NP, and CTX in male patients with T2DM treated with metformin (open circles), or pioglitazone (closed squares) Bars represent the 95% confidence interval

*: significance of change from baseline within groups (one-sample t-test), $p<0.05$

p-values: significance of differences between groups (student's t-tests)

Relationship between serum sclerostin and markers of bone turnover

There was no significant relationship between serum sclerostin levels and either serum P1NP (r=0.15, p=0.22) or serum CTX (r=0.17, p=0.17) at baseline. There was also no significant correlation between changes in serum sclerostin values and changes in serum P1NP levels in all patients pooled together (r=0.12, p=0.31), or in individual treatment groups (metformin: r=0.02, p=0.90; pioglitazone: r=0.05, p=0.77) (Figure 3A). In contrast, changes in serum sclerostin levels were significantly correlated with changes in serum CTX levels in all patients pooled together (r=0.36, p=0.002), and in pioglitazone treated patients (r=0.39, p=0.020), but not in the metformin treated patient

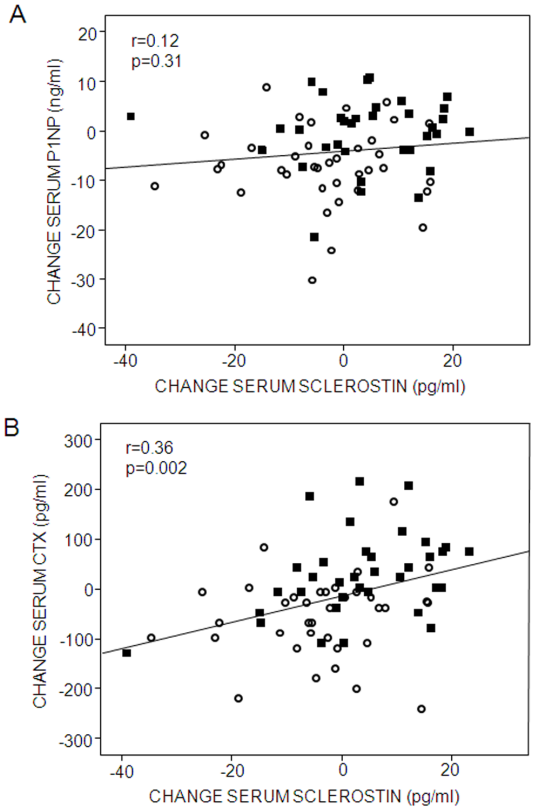

Figure 3. Relationship between changes of serum levels of sclerostin and P1NP (A) and CTX (B). Open circles represent metformin-treated patients, closed squares represent pioglitazone-treated patients

Discussion

We show here that circulating sclerostin levels are increased in patients with uncomplicated type 2 diabetes mellitus and respond differently to different blood glucose lowering medications. In the presence of similar control of glycaemia, metformin treatment given for 24 weeks had no effect on sclerostin levels and significantly decreased levels of biochemical markers of bone turnover, whereas pioglitazone treatment increased the serum levels of sclerostin and the bone resorption marker CTX.

The exact pathogenesis of increased bone fragility observed in patients with T2DM remains unclear. In these patients it is well established that fractures occur at higher bone mineral density (BMD) values than in patients with osteoporosis. The increased fracture rate has also been found to be independent of the increased frequency of falls, also documented in patients with T2DM (1, 14, 15). Changes in calcium homeostasis, increased secretion of inflammatory cytokines and the accumulation of advanced glycation end-products have all been proposed as contributory factors to the pathogenesis of increased bone fragility in T2DM (16, 17). The identification of osteocyte-produced sclerostin as a key regulator of bone formation by osteoblasts has initiated a number of studies of the effect of this protein on bone strength. Lack of sclerostin leads to profound increases in bone mass in humans (13, 18) and in animal models (19), whereas overexpression of sclerostin is associated with decreased mechanical strength in animal models (8). In a recent study circulating sclerostin levels were positively associated with fracture risk in a large cohort of postmenopausal women (9). Moreover, established determinants of bone strength such as mechanical loading, parathyroid hormone and estrogen have been shown to modulate the production and/or the secretion of sclerostin (12, 20, 21). Our study findings of increased serum sclerostin levels in patients with T2DM suggest a potential role for this protein in bone metabolism in T2DM by an as yet unidentified mechanism. The positive correlation between serum sclerostin and insulin levels suggests a possible contribution of insulin in the production and/or secretion of sclerostin. However, in contrast to the case of osteoblasts, few or no insulin receptors could be detected in osteocytes by immunohistochemical staining (22). In our

patients with T2DM the documented increase in serum sclerostin levels was not associated with a decrease in serum levels of the bone formation marker P1NP, in agreement with a previous study (23) . Another marker of bone formation osteocalcin, particularly the undecarboxylated form, has been implicated in the regulation of insulin secretion (24). However, measurements of serum osteocalcin in patients with T2DM have provided conflicting results showing either no change or decrease (23, 25-29). We did not measure serum osteocalcin but a significant positive relationship with serum P1NP has been previously reported in patients with T2DM (23, 28). Notwithstanding, our findings are in keeping with a recent study which reported higher serum sclerostin levels in patients with T2DM compared to controls but no difference in the bone formation markers serum osteocalcin and bone specific alkaline phosphatase between T2DM patients and healthy controls (26).

In our cohort of T2DM patients, treatment with metformin or pioglitazone in combination with glimepiride, improved glucose regulation to a similar degree, but had different effects on serum sclerostin levels and biochemical markers of bone turnover. Treatment with metformin for 24 weeks had no apparent effect on serum sclerostin levels, but significantly decreased bone turnover, as assessed by serum markers of bone formation (P1NP) and bone resorption (CTX). A decrease in bone turnover has been previously reported after metformin treatment (30, 31), but a similar decrease in bone turnover has also been noted after improvement of glycemic control using other therapeutic regimens, such as diet or insulin administration (32). However, the different effects on bone turnover observed in our study in the pioglitazone treated patients despite a similar control of glucose metabolism demonstrate that the two agents have different effects on bone metabolism that are independent of their glucose-lowering action.

Our data indeed show that in contrast to metformin, serum sclerostin levels increased in patients treated with pioglitazone and that this was associated with a significant increase in serum CTX levels, despite adequate glycemic control. Over the past decade evidence has been accumulating on the detrimental effect of thiazolidinediones (TZDs) on the skeleton, decreasing bone mass and increasing fracture risk in both men (33-35) and women with diabetes mellitus (4, 33, 35-39). This deleterious effect of TZDs on the skeleton is generally attributed to the activation of PPAR-γ in the

bone marrow by these agents, leading to preferential stimulation of adipogenesis at the cost of osteoblastogenesis (5). Our results suggest another potential pathogenetic mechanism, namely stimulation of sclerostin production by TZDs. This finding conforms with recent *in vitro* data by Mabbilieau et al. who showed that TZDs stimulate the expression of sclerostin by osteocytes in the absence of 17β-estradiol (6). Although sclerostin is a well established inhibitor of bone formation, recent evidence indicates that it can also promote osteoclastogenesis by stimulating RANKL produced by osteocytes (40). Moreover, inhibition of sclerostin in animals and humans by a specific antibody does not only lead to increased bone formation but also to decreased bone resorption (41, 42). Taken together, these data suggest that pioglitazone stimulates the production of sclerostin, which would in turn increase RANKL production by osteocytes, thus resulting in a dual effect on bone metabolism, reducing bone formation and increasing bone resorption, which could explain the adverse effect of pioglitazone on bone quality.

A limitation of our study is that it was conducted only in men with uncomplicated T2DM and our results cannot be readily extrapolated to all patients with T2DM. The study was of short duration precluding the assessment of the effect of TZDs on BMD or fracture risk

Notwithstanding, our study shows that patients with T2DM have increased circulating sclerostin levels which might contribute to the documented increased bone fragility of these patients. Of particular clinical relevance is the finding that metformin and pioglitazone have different effects on sclerostin levels and biochemical markers of bone turnover, with metformin having a clearly more favorable bone profile.

Funding

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Chapter 9

Serum sclerostin levels in Paget's disease and prostate cancer with bone metastases with a wide range of bone turnover

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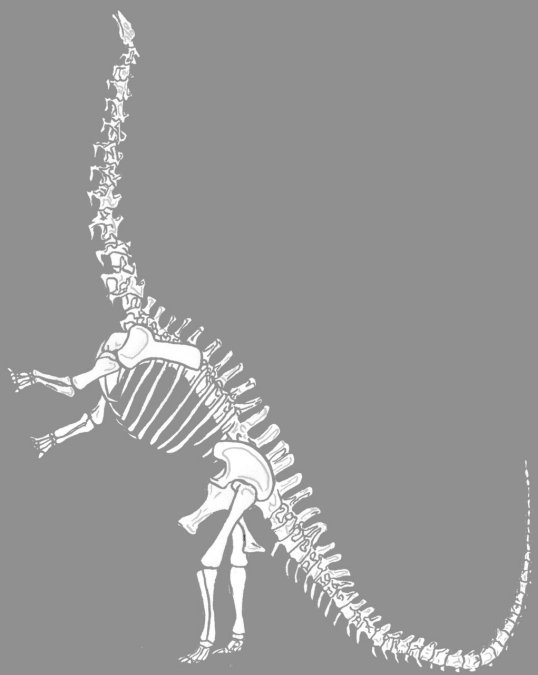

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Abstract

Evidence has been accumulating for the role of osteocytes as key players in the regulation of bone remodeling. One of the main products of these cells, sclerostin, inhibits bone formation and may also stimulate bone resorption. Circulating sclerostin has been evaluated in humans, but data are scarce in patients with different rate of bone turnover. To address this issue we evaluated serum sclerostin levels in patients with Paget's disease of bone (PD) and in patients with prostate cancer metastatic to the skeleton (PC).

Sclerostin levels were measured in 88 patients with PD, 20 patients with PC and 237 healthy individuals (113 men and 124 women, aged 20 to 77). Bone turnover was evaluated by measuring serum levels of procollagen type 1 amino-terminal propeptide (P1NP) in all individuals studied and β-carboxy-terminal cross-linking telopeptide of type I collagen (β-CTX) only in patients. Patients were aged between 45 and 88 years and had a wide range of bone turnover: serum P1NP 9.2 to 1872 ng/ml and β-CTX 50 to 3120 pg/ml. Patients with PD and with PC had significantly higher mean serum sclerostin levels (53.1 ± 22.7 pg/ml and 56.6 ± 25.8 pg/ml, respectively) compared to healthy controls (38 ± 12.1 pg/ml) ($p<0.001$). Serum sclerostin levels were significantly correlated with P1NP in all ($n=345$) studied subjects ($r=0.32$, $p<0.001$) Circulating sclerostin levels are significantly increased in patients with increased bone turnover, regardless of underlying pathology. These increased levels may be due to a compensatory response to the increased number of osteoblasts at affected skeletal sites and may contribute to the increased bone resorption in patients with PC .

Introduction

Osteocytes are former osteoblasts embedded in lacunae of the mineralized bone matrix. Over the last decade evidence has unfolded about the critical role of these abundant bone cells as orchestrators of bone remodeling through regulation of both osteoblast and osteoclast activity, and as endocrine regulators of phosphate homeostasis [1]. Osteocytes communicate with each other and with other cellular components of bone through an extensive network of cytoplasmic processes, thereby fine tuning the differentiation and function of these cells.

One of the main secreted products of osteocytes, sclerostin, is solely expressed by mature cells and is considered a major negative local regulator of bone formation [2, 3] . This takes place primarily through binding with the low-densitity lipoprotein receptor-related protein 5 and 6 (LRP5/6) co-receptors and consequently inhibiting the canonical Wnt signaling pathway [4, 5]. Sclerostin has attracted much attention over the last few years due to its implication in the pathogenesis of two rare skeletal disorders characterized by substantial increase in bone mass, sclerosteosis and van Buchem disease [6-9]. In addition, suppression of sclerostin production is, at least partly, responsible for the anabolic effect of mechanical loading and PTH on bone [10-12].

Apart for its well documented role as an inhibitor of bone formation, sclerostin is also involved in the regulation of bone resorption. Recent evidence indicates that osteocytes are the main source of RANKL in the skeleton [13, 14] and that sclerostin promotes osteoclast formation and activity by osteocytes in a RANKL-dependent manner [15]. Moreover, a neutralizing antibody against sclerostin increased significantly the rate of bone formation in animal models while it did not change or decreased bone resorption, assessed histomorphometrically [16, 17]. Finally, in a phase 1 clinical trial a single parenteral administration of an antibody to sclerostin led to a rapid and dose-dependent increase in serum P1NP and decreased serum CTX [18].

To obtain more insight into the relationships between sclerostin and bone remodeling in humans, we evaluated serum sclerostin levels in patients with Paget's disease of bone, characterized by predominantly increased bone resorption, at different stages of disease activity, and in patients with prostate cancer metastatic to the skeleton mainly characterized by increased bone formation, at different degrees of skeletal involvement.

Material and Methods

Study population

Eighty eight patients with biochemical, radiological and scintigraphic evidence for Paget's disease of bone and 20 patients with histological and scintigraphic evidence for prostate cancer metastatic to the skeleton, who were under regular control in the Outpatient Clinic of the Department of Endocrinology and Metabolic Diseases of the Leiden University Medical Center were included in the study. Exclusion criteria were impaired renal function (serum creatinine >120 μmol/l), impaired liver function, or the presence of diseases known to increase bone turnover other than Paget's disease of bone and prostate cancer. Stored at -80°C and previously unthawed serum samples were measured for sclerostin, the bone formation marker procollagen type 1 amino-terminal propeptide (P1NP) and the bone resorption marker β-carboxy-terminal cross-linking telopeptide of type I collagen (β-CTX).

Subject characteristics

Of the 88 patients with Paget's disease of bone, 47 had monostotic (predominantly pelvis) and 41 had polyostostic disease (ranging from 2 to 8 affected skeletal sites). Twenty four patients were treatment–naïve at the time of sampling and 64 had been previously treated with one or more (median 2) courses of bisphosphonates (short courses of intravenous pamidonate 45, olpadronate 46, EB-1053 4, zoledronate 1; monthly courses of oral olpadronate 3 , oral etidronate 8) Twenty eight of these patients had received treatment within the last 4 years, and 36 received their last course of treatment more than 5 years before the time of sampling (range 5 to 25 years). All patients with metastatic prostate cancer were androgen-deprived and bisphosphonate-treatment naïve at time of sampling.

Serum sclerostin levels obtained from patients were compared with those obtained from two cohorts of healthy individuals, who were used as controls. The first, previously described, cohort consisted of 72 healthy volunteers, 29 males and 43 females, with a mean age of 50.4 years (range: 20-77 years) [19] . The second cohort consisted of 165 healthy individuals, 84 males and 81 females, with a mean age of 65.1 years (range 63-68 years) studied in collaboration with the Division of Bone Diseases of the University of Geneva [20] (20). All subjects had normal renal function and had

serum P1NP values below 65 ng/ml.
The study was approved by the Medical Ethics Committee of the Leiden University Medical Center and the Geneva University Hospitals and informed consent was obtained from all participants.

Biochemical assays
Serum calcium, phosphate and creatinine were measured by semi-automated techniques. Serum alkaline phosphatase activity (ALP) was measured using a fully automated P800 modulator system (Roche BV) and 25-hydroxyvitamin D (25-OHD) by the Liason 25-OHD assay (DiaSorin SA). Plasma PTH was measured by limulite 2500 (Siemens Diagnostics). Serum P1NP and β-CTX were determined using the E-170 system (Roche BV).

Sclerostin assay
Sclerostin was measured in serum by an electrochemiluminescence assay (MSD 96-well MULTI-ARRAY Human Sclerostin Assay, Gaithersburg, MD, USA), as previously described [21]. In brief, the sclerostin standard for the assay was produced in an NSO-derived myeloma cell line, and was checked for purity by SDS–PAGE gel with silver stain. The intra- and inter–assay coefficients of variation were 6% and 10% respectively. The detection limit of the assay was 1 pg/ml, and the detection range was 1–10 000 pg/ml. Using this assay, sclerostin was detected in the serum of all healthy subjects and was undetectable in the serum of 19 patients with sclerosteosis [21].

Statistical analysis
Data are expressed as mean ± Standard Deviation (SD). Normality of distribution was assessed by Kolmogorov–Smirnov test and log-transformation of data was applied for non-normally distributed variables. Student's t-test for independent samples or One-way ANOVA was used for differences between groups, as applicable. Pearson's X^2 test was applied to explore associations between sclerostin values and bone markers in serum. A probability level of random difference of 0.05 was considered significant. Data were analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

Results

The patient population studied consisted of 108 subjects (75 men and 33 women) with a mean age of 68.1 years (range 45 to 88 years). Demographic characteristics and laboratory values are shown in Table 1. As intended by study design, there was a wide range of bone turnover within the patient population, with serum P1NP ranging from 9.2 to 1872 ng/ml (mean 223.3 ng/ml) and β-CTX ranging from 50 to 3120 pg/ml (mean 642.0 pg/ml) (Figure 1). There was a significant correlation between serum levels of β-CTX and P1NP in the whole patient population studied ($r=0.78$, $p<0.001$), as well in the two separate patient groups (Paget's disease of bone: $r=0.69$, $p=<0.001$, bone metastatic disease: $r=0.86$, $p<0.001$).

Table 1. Demographic characteristics and baseline laboratory values of patients

	Paget's disease of bone	Metastatic prostate cancer	Controls	Reference range
Number	88	20	237	-
Male : female	55:33	20:0	113:124	-
Age (yrs)	67.4 ± 10.6	71.1 ± 7.3	60 ± 11.3	-
BMI (kg/cm^2)	27.6 ± 4.1	26.4 ± 3.7	25.1 ± 4.3	-
P1NP (ng/ml)	158 ± 235	511 ± 441	35.6 ± 12.7	< 65
ALP (U/l)	196 ± 248	834 ± 900	65.3 ± 16.3[1]	40 – 120
β-CTX (pg/ml)	498 ± 330	1,275 ± 806	-	< 590
Calcium (mmol/l)	2.37 ± 0.14	2.26 ± 0.12	2.27 ± 0.08[1]	2.15 – 2.55
Phosphate (mmol/l)	1.05 ± 0.17	1.09 ± 0.27	1.07 ± 0.15	0.90 – 1.50
Creatinine (μmol/l)	88.4 ± 13.5	81.2 ± 16.6	77 ± 15	44 – 120
25-OH D (nmol/l)	58.6 ± 28.6	55.3 ± 24.9	-	> 50
PTH (pmol/l)	6.6 ± 3.6	5.0 ± 1.6	-	1.5 -8.0

[1]Values obtained in a subset of the sohort (LUMC)

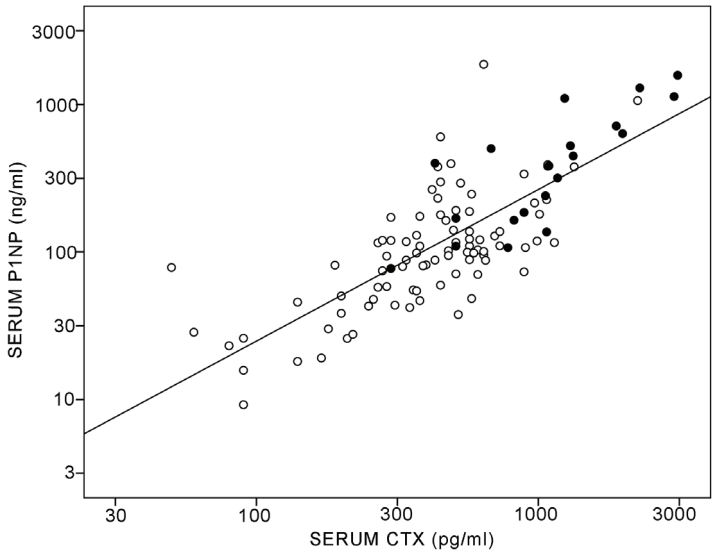

Figure 1. Serum levels of P1NP and β-CTX in patients with Paget's disease of bone, and prostate cancer with bone metastasis. Open circles Paget's disease of bone, closed circles bone metastatic disease from prostate cancer. ($r=0.78$, $p<0.001$).

Circulating sclerostin levels

In patients, mean serum sclerostin level was 53.8 pg/ml (range 11.9 pg/ml to 135.2 pg/ml) and was significantly higher ($p<0.001$) than the mean value of sclerostin of all 237 healthy controls (38.1 pg/ml; range 12.4 pg/ml to 80.1 pg/ml) (Figure 2). In both patients and healthy individuals there was no correlation between serum sclerostin levels and age.

Paget's disease of bone

In patients with Paget's disease sclerostin levels ranged from 11.9 to 135.2 pg/ml, with the mean level being significantly higher than that of healthy controls (53.1 pg/ml vs 38.0 pg/ml respectively, $p<0.001$). Because of the predominance of elderly men in the Paget's cohort, results were also analyzed according to gender and age (younger or older than 65 years). Results showed that the difference in sclerostin values remained robust (Table 2) There was no significant difference in mean levels of

sclerostin between previously bisphosphonate-treated and bisphosphonate-treatment naïve patients (55.2 pg/ml vs. 47.5 ng/ml respectively, p=0.16), while these were higher in patients with polyostotic compared to monostotic disease (59.0 pg/ml vs. 48.1 pg/ml, p=0.024).

Prostate cancer metastatic to the skeleton

In patients with prostate cancer metastatic to the skeleton serum sclerostin levels ranged from 19.4 pg/ml to 120.5 pg/ml, with a mean level significantly higher than that of healthy controls (57.6 vs. 38.1 pg/ml, respectively, p=0.005). This difference remained significant when values of patients were compared to those of healthy males or males older than 65 years (Table 2). There was no difference in mean sclerostin levels between patients with prostate cancer metastatic to the skeleton and those with Paget's disease of bone (p=0.55).

Relationship between sclerostin levels and biochemical markers of bone turnover.

There was a significant relationship between serum sclerostin and serum P1NP in studied subjects (n=345, r=0.31, p<0.001). In patients with Paget's disease alone, there was no relationship between serum sclerostin and P1NP levels (r=0.08, p=0.45). In contrast, in patients with prostate cancer serum levels of sclerostin were significantly correlated with those of P1NP (r=0.56,. p=0.01), Circulating sclerostin levels were not correlated with levels of β-CTX in the whole patient population (r=0.16, p=0.09), or in patients with Paget's disease (r=0.08, p=0.46), but were correlated in patients with prostate cancer (r=0.48, p=0.03).

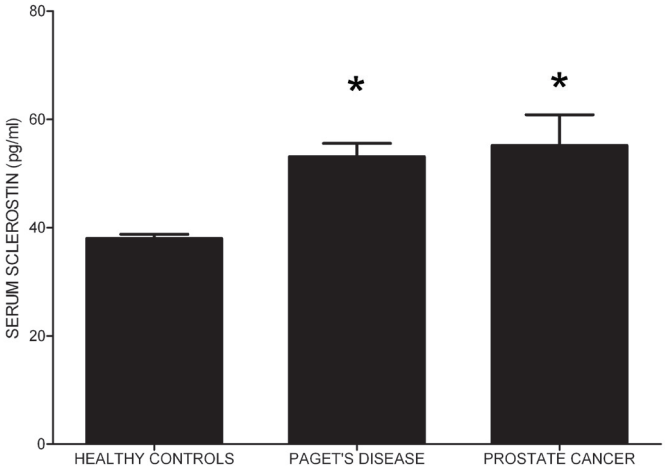

Figure 2. Serum sclerostin levels in healthy controls, and patients with Paget's disease of bone, and prostate cancer with bone metastasis. *: $p<0.01$ compared to healthy controls

Table 2. Serum sclerostin in healthy individuals and patients with Paget's disease and prostate cancer metastatic to bone.

Subjects	all	>65years	men	men >65 years
Healthy	38.1 ± 12.1	38.3 ± 9.9	40.2 ± 12.5	39.8 ±10.2
	(n=237)	(n=102)	(n=113)	(n=55)
Paget's disease	53.1 ±22.7*	53.5 ± 25.3*	55.4 ± 24.1*	55.8 ± 27.6*
	(n=88)	(n=54)	(n=55)	(n=33)
Prostate cancer	56.6 ± 25.8*	54.9 ± 22.8*	56.6 ± 25.8*	54.9 ± 22.8*
	(n=20)	(n=16)	(n=20)	(n=16)

* $p<0.05$ compared to healthy controls

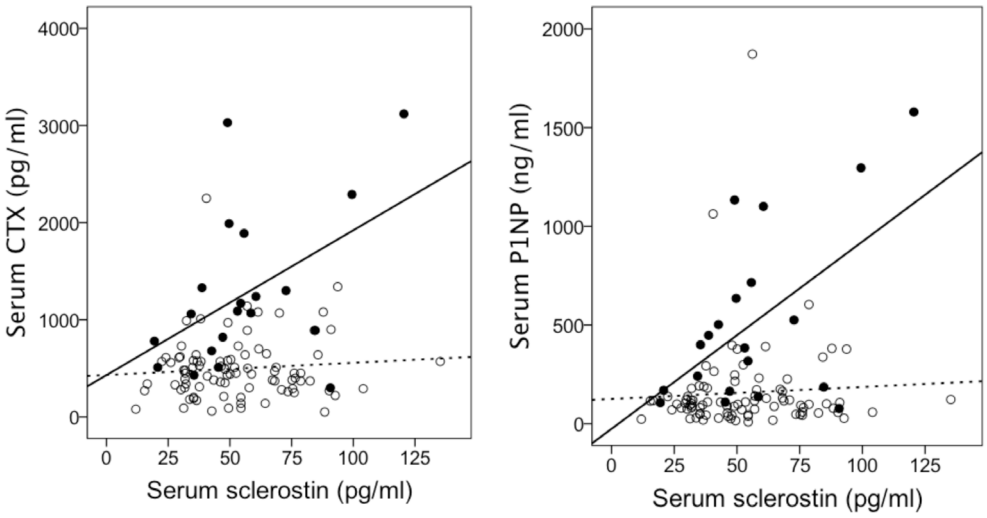

Figure 3. Relationship between serum levels of sclerostin and P1NP (A) and sclerostin and CTX (B) in patients with Paget's disease of bone (open circles and solid line) and prostate cancer with bone metastasis (closed circles and interrupted line).

Discussion

Sclerostin is an osteocyte-produced local regulator of bone remodeling affecting both osteoblast and osteoclast function [2, 3, 15]. A previous study has shown that there is a strong relationship between circulating and bone marrow plasma levels of sclerostin consistent with the findings that osteocytes are the major source of sclerostin production and, therefore, the sclerostin present in peripheral blood [22]. Our data show that circulating levels of sclerostin are significantly increased in diseases associated with increased bone turnover, such as Paget's disease and prostate cancer metastatic to the skeleton, regardless of the underlying pathogenetic mechanism responsible for the increased bone turnover.

In Paget's disease of bone, bone resorption is increased and is followed by increased and disorganized bone formation. In prostate cancer, skeletal metastases are mainly osteoblastic but increased bone resorption has also been biochemically, as also

shown here, and histologically documented [23-27]. Despite the difference in the pathogenetic mechanisms of Paget's disease of bone and prostate cancer metastatic to the skeleton, both disorders share the common end-result of increased bone turnover. We found increased circulating sclerostin levels in both diseases compared to healthy controls which were independent of renal function, age and previous bisphosphonate treatment. All subjects included in our study had normal renal function and it was recently shown that serum sclerostin levels increase sharply in subjects with renal impairment (CKD 3 stage) [28]. We did not find any association of serum sclerostin levels with age consistent with some [29-33] but not other [28, 34-38] reports which may reflect differences in the characteristics of studied cohorts and/or specificity of sclerostin assays. Finally, previous bisphosphonate treatment of patients with Paget's disease had no effect on sclerostin levels which may be due to inability of used bisphosphonates to reach the osteocytes and/or the long interval following the short exposure to the agents in our study. In orevious studies of patients with osteoporosis serum sclerostin did not change during treatment with alendronate [32] while they increased during treatment with risendronate or niridronate given intramuscularly [30, 39].

We found, in addition, a significant positive relationship between sclerostin levels and bone turnover markers in metastatic prostate cancer but not in Paget's disease of bone. Although this result may reflect the difference in underlying pathophysiology between the two disorders, it remains difficult to draw any conclusion about the exact mechanism which regulates sclerostin production in these conditions. We could speculate, however, that in both diseases osteocytes may secrete more sclerostin as a compensatory response to the increased number of osteoblasts that are recruited to the affected skeletal sites.

A number of factors could stimulate sclerostin production by osteocytes in either disorder. It has recently been demonstrated that, Dkk-1, an antagonist of Wnt signaling, is also significantly elevated in the serum of patients with Paget's disease of bone [40]. Dkk-1 expression and protein concentration have also been reported to be elevated in osteoblastic and stromal cell cultures from Pagetic lesions [41] In addition, Dkk-1 has been found to positively regulate sclerostin expression and to enhance suppression of the Wnt signaling pathway in inflammatory–induced bone loss, so that an increased production of Dkk-1 by Pagetic lesions could also be

responsible for increased sclerostin production by osteocytes [42]. Cytokines produced by prostate cancer cells, such as BMP-6 [43, 44], which is a known stimulator of sclerostin production [45, 46], could also contribute to the increased circulating levels of sclerostin observed in prostate cancer metastatic to the skeleton. This increased sclerostin production may in turn stimulate RANKL and bone resorption. Sclerostin was recently shown to upregulate the expression of RANKL by osteocyte-like cells and to promote osteoclastogenesis [15], supporting also the view that osteocytes are targets for sclerostin action. This sequence of events can explain the association between serum sclerostin levels with those of P1NP and CTX observed in patients with prostate cancer metastatic to the skeleton. The same mechanism may not be operational in Paget's disease in which RANKL is already upregulated [47].
In conclusion we demonstrate increased circulating sclerostin levels in two skeletal disorders characterized by increased bone turnover, regardless of their underlying pathogenetic mechanisms. These increases in serum sclerostin may be due to a compensatory response to the increased number of osteoblasts at affected skeletal sites and may contribute to increased bone resorption in patients with prostate cancer with bone metastases.

Ackowledgements

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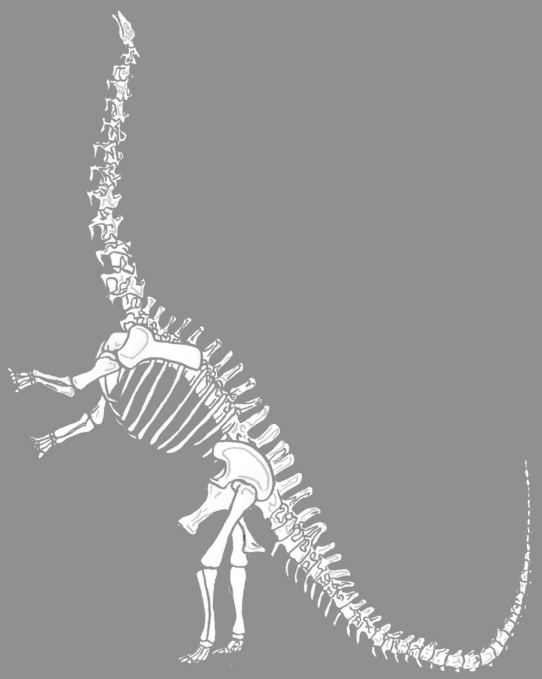
Summary & Conclusions



Summary and Conclusions

During the last decade the osteocyte-derived protein sclerostin has emerged as a key regulator of bone remodeling. As described in **Chapter 1**, sclerostin acts as an inhibitor of the canonical Wnt pathway in osteoblasts, thereby inhibiting bone formation. In patients with sclerosteosis and van Buchem disease impaired sclerostin synthesis leads to unrestrained bone formation, and a phenotype of generalized hyperostosis with secondary complications due to overgrowth of the skull bones. The synthesis of sclerostin is controlled by systemic and local factors, and aberrant sclerostin synthesis is thought to be associated with several bone disorders other than sclerosteosis and van Buchem disease. Because the production and function of sclerostin is restricted to the skeleton, it presents an attractive target for bone building therapies for osteoporosis. The effects of neutralizing antibodies against sclerostin on bone mass and strength have been investigated in animal models and are currently studied in clinical trials in humans.

The main objective of this Thesis was the investigation of the regulation of production of sclerostin and its effect on bone metabolism in humans. For this, we measured circulating sclerostin by an electrochemiluminescence assay developed by Meso Scale Discovery Inc. The characteristics, specificity and sensitivity of this assay are described in **Chapter 2.** We determined the specificity of the assay for sclerostin by epitope mapping, and we tested the reactivity of sclerostin fragments of different lengths. Results showed that the assay is specific for the entire sclerostin molecule and sufficiently accurate for use in clinical studies.

In **Chapter 3** we describe a study of 19 South African patients with sclerosteosis and 26 heterozygous carriers of the disease. The purpose of this study was to determine the effect of impaired synthesis of sclerostin on the phenotype and bone turnover of these individuals. We show that there is a gene-dose effect of the slerosteosis mutation on circulating sclerostin, with absent sclerostin in serum of patients and decreased serum sclerostin levels in disease carriers, associated with normal phenotype in the latter. These differences in circulating sclerostin were accompanied by different levels of the bone formation marker serum P1NP between patients, carriers and healthy

controls. These results provide in vivo evidence of different degrees of increased bone formation caused by the absence or decreased synthesis of sclerostin in humans. They also suggest that inhibition of sclerostin can be titrated, since the decreased sclerostin levels in disease carriers did not lead to any of the symptoms or complications of the disease but had a positive effect on bone mass.

In contrast to sclerosteosis, the *SOST* gene is unaffected in patients with van Buchem disease who lack a regulatory element for the transcription of the gene due to a 52kb deletion. In **Chapter 4,** we describe a study of 15 Dutch patients with van Buchem disease and 28 disease carriers and we show that patients produce small amounts of sclerostin. This finding conforms with the previously reported and confirmed in our study milder phenotype of the disease compared to that of sclerosteosis. Carriers of van Buchem disease had lower sclerostin levels than healthy controls, but the overlap with the levels of healthy controls was larger than that found in carriers of sclerosteosis. As a result of that, mean levels of serum P1NP although higher in carriers compared to controls, were not significantly different.

An important and clinically relevant finding of the studies of sclerosteosis and van Buchem disease described in **Chapters 3** and **4** was the stabilization of the bone complications of these diseases in adulthood. This finding was paralleled by a decline in serum P1NP levels with age in both patients with sclerosteosis and van Buchem disease, and the stabilization of bone mineral density in patients with van Buchem disease. These results strongly suggest that sclerostin is especially critical for bone formation during the first two decades of life.

No medical treatment is available for either sclerosteosis or van Buchem disease and current management of the complications is surgical, aiming at removal of the excess of bone, a technically difficult and sometimes dangerous procedure. Glucocorticoids are known inhibitors of bone formation and we hypothesized that administration of these agents to patients with complications due to bone overgrowth may arrest their progress. In **Chapter 5** we present long-term observations of a patient with van Buchem disease with life-threatening increased intracranial pressure treated with prednisone. Treatment with prednisone resulted in biochemically and histologically documented suppression of bone formation and arrest of the progression of bone

accumulation. These results suggest that glucocorticoids may offer an alternative treatment to the high risk surgical approaches used in the management of patients with progressive sclerosing bone diseases. Our findings also suggest that whereas sclerostin may not be required for the action of glucocorticoids on bone formation, it may well be important for the action of these agents on bone resorption.

Glucocorticoid use is associated with deleterious effects on bone, particularly bone formation, leading to glucocorticoid-induced osteoporosis, a common cause of secondary osteoporosis. In mice glucocorticoids increase the expression of the *SOST* gene suggesting a role for sclerostin in the pathogenesis of bone fragility. To assess whether glucocorticoids stimulate sclerostin synthesis in humans, in **Chapter 6** we measured circulating sclerostin in patients with Cushing's disease and endogenous hypercortisolism**.** Compared to healthy controls, these patients had significantly lower plasma sclerostin levels which increased after treatment of the disease. Thus, contrary to our hypothesis, sclerostin does not appear to play a role in the effect of chronic hypercortisolism on bone formation. Sclerostin is almost exclusively expressed by osteocytes, cells that are directly susceptible to the effect of glucocorticoids, which promote their apoptosis. We, therefore, investigated the possibility that the decreased sclerostin levels in patients with endogenous hypercortisolism may be due to a direct effect of glucocorticoids on osteocytes. For this, we measured plasma levels of fibroblast growth factor 23 (FGF23), which is also produced by osteocytes. Similar to sclerostin, FGF23 levels significantly increased during biochemical remission of the disease suggesting that the low sclerostin levels of patients with increased endogenous production of cortisol are due to a decrease in the number or function of the osteocytes.

In vitro and *in vivo* studies in animal models have shown that parathyroid hormone (PTH) inhibits the expression of the *SOST* gene and/or sclerostin. In **Chapter 7** we tested the hypothesis that chronic PTH excess decreases circulating sclerostin in humans. For this**,** we studied 25 patients with elevated serum PTH concentrations due to primary hyperparathyroidism (PHPT) and 49 patients cured from PHPT by successful parathyroidectomy. We found that patients with PHPT have significantly lower serum sclerostin levels compared to patients with normal PTH concentrations

following parathyreoidectomy. The observed negative correlation between PTH and sclerostin levels in these individuals strongly suggests that *SOST* is downregulated by PTH in humans.

Patients with Type 2 Diabetes Mellitus (T2DM) have an increased risk of fractures and thiazolidinediones (TZDs) increase this risk. Because TZDs stimulate the expression of sclerostin *in vitro*, abnormal sclerostin production may be involved in the pathogenesis of increased bone fragility in patients with T2DM treated with TZDs. In **Chapter 8** we show that men with uncomplicated T2DM have significantly higher serum sclerostin levels than healthy controls and that these levels increase after treatment with the TZD pioglitazione and are accompanied by increases in the levels of the bone resorption marker CTX. These findings suggest that increased sclerostin production may be involved in the pathogenesis of skeletal fragility in patients with T2DM in general, and may specifically contribute to the detrimental effect of TZDs on bone. In contrast, metformin treatment has no effect on serum sclerostin levels and it reduces bone turnover having, thus, a more favourable effect than pioglitazone on bone metabolism.

In the previous **Chapters** we showed that decreased sclerostin synthesis leads to an increase in bone formation but may also stimulate bone resorption under certain conditions. In **Chapter 9** we, therefore, examined the relation between sclerostin and bone turnover in diseases characterized by increased bone turnover. For this, we measured serum sclerostin levels in patients with Paget's disease of bone, characterized by predominantly increased bone resorption, at different stages of disease activity, and in patients with prostate cancer metastatic to the skeleton mainly characterized by increased bone formation, at different degrees of skeletal involvement. Compared to healthy individuals, mean serum sclerostin levels were significantly higher in both disorders and were positively associated with levels of serum P1NP, due possibly to a compensatory response to the increased number of osteoblasts at affected skeletal sites. Furthermore, sclerostin levels were significantly associated with serum levels of CTX in patients with prostate cancer metastatic to the skeleton, but not in patients with Paget's disease of bone and may contribute to the increased bone resorption in

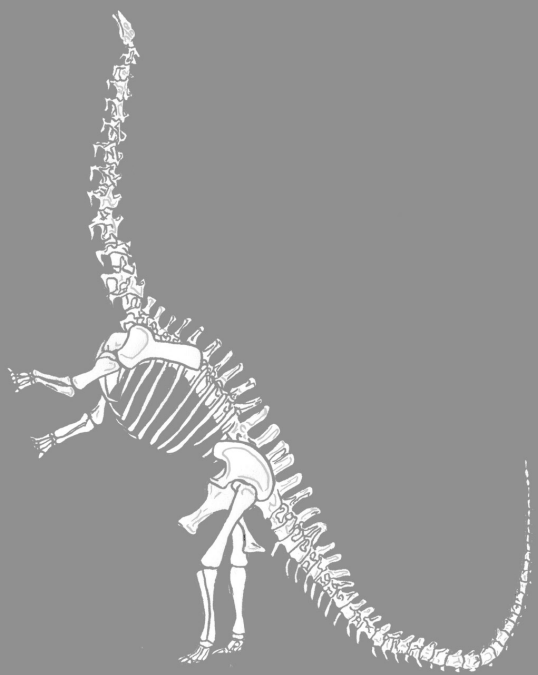
patients with prostate cancer metastatic to the skeleton.

Concluding remarks

The described dramatic skeletal phenotypes of sclerosteosis and van Buchem disease underscore the essential role of sclerostin in the regulation of bone formation, while our findings in carriers of these disorders indicate that milder deficiencies in sclerostin synthesis are also associated with increased bone formation but do not to lead to complications. The combined results of these studies fully support the rationale of the use of sclerostin inhibitors as bone building agents in patients with osteoporosis. However, the lack of progression of skeletal complications in both sclerosteosis and van Buchem disease in adulthood, and the decline in serum P1NP levels with age in patients and carriers, raises questions about the kinetics of bone remodelling (transient or continuous) to long-term exposure of elderly individuals to inhibitors of sclerostin.

During the investigations described in this Thesis, new data became available indicating that sclerostin does not only inhibit bone formation, but can also stimulate bone resorption by a RANKL-mediated mechanism in osteocytes. Some of our results are fully consistent with these findings and help to explain the complex action of sclerostin in humans. Whether measurement of circulating sclerostin can be of use in clinical practice is uncertain, due to the substantial overlap of levels between healthy subjects and patients and its dependence on numerous modulators. On the other hand, it is also clear that sclerostin measurements provide for a very important tool for the assessment of the function of osteocytes on bone metabolism in humans. The present and previously reported studies illustrate the importance of osteocytes in the regulation of bone metabolism and underscore the role of sclerostin as a key determinant of bone remodelling and strength in humans.

Samenvatting & Conclusies



Samenvatting en conclusies

Gedurende de laatste tien jaar is duidelijk geworden dat het door osteocyten geproduceerde eiwit sclerostine een belangrijke regulator is van botombouw. Zoals beschreven in **hoofdstuk 1** werkt sclerostine als een antagonist van de Wnt signaleringsroute in osteoblasten, waarmee het botformatie door deze cellen remt. In patiënten met sclerosteose en de ziekte van Van Buchem leidt een gereduceerde sclerostine synthese tot ongeremde botformatie, en een fenotype van gegeneraliseerde hyperostose met secundaire complicaties door botwoekering in de schedel. De synthese van sclerostine wordt gecontroleerd door systemische en lokale factoren, en een afwijkende sclerostinesynthese lijkt geassocieerd te zijn met verscheidene skeletaandoeningen anders dan sclerosteose en de ziekte van Van Buchem. Omdat de productie en functie van sclerostine beperkt is tot het skelet, is het een aantrekkelijk aangrijpingspunt voor botopbouwende therapieën voor osteoporose. Het effect van neutraliserende antilichamen tegen sclerostine op botmassa en -sterkte is onderzocht in verscheidene diermodellen en wordt momenteel in klinische studies in mensen bestudeerd.

De doelstelling van dit proefschrift was het bestuderen van de regulatie en productie van sclerostine en het effect van dit eiwit op botmetabolisme in mensen. Hiervoor hebben wij circulerend sclerostine gemeten met een elektrochemiluminiscentie bepaling ontwikkeld door *Meso Scale Discovery Inc.* De karakteristieken, specificiteit en sensitiviteit van deze analyse staan beschreven in **hoofdstuk 2**. Voor het vaststellen van de specificiteit van de bepaling voor sclerostine hebben we de epitopen in kaart gebracht, en de reactiviteit van sclerostinefragmenten van verschillende lengtes bestudeerd. De desbetreffende resultaten laten zien dat de analyse specifiek is voor het gehele sclerostinemolecuul en accuraat genoeg voor het gebruik in klinische studies.

In **hoofdstuk 3** beschrijven we een studie van 19 Zuid-Afrikaanse patiënten met sclerosteose en 26 heterozygote dragers van de ziekte. Deze studie had als doel te onderzoeken welk effect een verminderde sclerostineproductie heeft op het fenotype en de botombouw van deze individuen. We laten zien dat er een gen-dosis effect is van de sclerosteosis mutatie op circulerende sclerostinewaarden, met absentie van sclerostine

in het serum van patiënten en verminderde serum sclerostinewaarden in dragers van de ziekten, waarbij laatsgenoemden een normaal fenotype hebben. De verschillen in sclerostinewaarden gingen gepaard met verschillen in de waarden van de marker voor botformatie P1NP tussen patiënten, dragers en gezonde controles. Deze resultaten leveren *in vivo* bewijs voor graduele veranderingen in de mate van botformatie door absentie, dan wel verminderde synthese van sclerostine. Dit suggereert ook dat de inhibitie van sclerostine kan worden getitreerd, aangezien de verminderde sclerostine synthese in dragers niet leidde tot enige symptomen of complicaties van sclerosteose, maar wel een positief effect op botmassa had.

In tegenstelling tot sclerosteose is in patiënten met de ziekte van Van Buchem het *SOST* gen onaangetast. Deze patienten missen echter een regulerend element voor de transcriptie van het gen door een 52kb deletie op chromosoom 17. In **hoofdstuk 4** beschrijven wij een studie van 15 Nederlandse patiënten met de ziekte van Van Buchem en 27 dragers van de ziekte. Wij laten onder andere zien dat patiënten geringe hoeveelheden sclerostine produceren. Deze bevinding is in lijn met het eerder gerapporteerde, en in onze studie bevestigde, mildere fenotype van deze ziekte ten opzichte van dat van sclerosteose. Dragers van de ziekte van Van Buchem hadden lagere sclerostinewaarden dan gezonde controles, maar de overlap met de waarden van gezonde controles was groter in vergelijking met die gevonden in dragers van sclerosteosis. Zodoende waren de gemiddelde waarden van serum P1NP wel hoger in dragers dan controles, maar niet significant verschillend.

Een belangrijke en klinisch relevante bevinding van de studies naar sclerosteose en de ziekte van Van Buchem, beschreven in **hoofdstuk 3 en 4,** was de stabilisatie van complicaties van de ziekten in volwassenheid. In lijn met deze bevinding was de afname van de waarden van P1NP met de leeftijd in patiënten met sclerosteose en de ziekte van Van Buchem. De resultaten suggereren in sterke mate dat sclerostine met name van groot belang is voor de regulatie van botformatie gedurende de eerste twee decennia van het leven.

Er zijn geen medicinale behandelingen voor sclerosteose of de ziekte van Van Buchem beschikbaar. De behandeling van complicaties is chirurgisch, gericht op het verwijderen van overtollig bot, een technisch ingewikkelde en soms gevaarlijke

procedure. Glucocorticosteroïden zijn bekende remmers van botformatie en wij beredeneerden dat de toediening van deze medicijnen aan patiënten met complicaties ten gevolge van botwoekering de progressie hiervan kon remmen. In **hoofdstuk 5** presenteren we langetermijn observaties van een patiënt met de ziekte van Van Buchem met levensbedreigende verhoogde intracraniële druk die werd behandeld met prednison. Behandeling met prednison resulteerde in biochemisch en histologisch vastgestelde onderdrukking van de botformatie en een arrest van verdere progressie van botaccumulatie. Deze resultaten suggereren dat glucocorticosteroïden als een alternatieve therapie kunnen dienen voor de risicovolle chirurgische ingrepen waarop de behandeling van patiënten met progressieve sclerotiserende botaandoeningen momenteel is gestoeld. Onze bevindingen suggereren ook dat alhoewel sclerostine niet van belang is voor de invloed van glucorticosteroïden op botformatie, het mogelijk wel een rol speelt bij het effect van deze hormonen op botresorptie.

Het gebruik van glucorticosteroïden heeft een negatief effect op het bot, waarbij met name botformatie is aangedaan. Glucocorticosteroïd-geïnduceerde osteoporose is dan ook een veelvoorkomende oorzaak van secundaire osteoporose. Glucocorticoïden verhogen in muizen de expressie van het *SOST* gen, hetgeen een rol van sclerostine suggereert in de pathogenese van botfragiliteit. Om te onderzoeken of glucocorticosteroïden ook in mensen sclerostinesynthese stimuleert, hebben we circulerend sclerostine gemeten in patiënten met de ziekte van Cushing en endogeen hypercortisolisme, zoals beschreven in **hoofdstuk 6**. Deze patiënten hadden in vergelijking met gezonde controles significant lagere sclerostinewaarden, welke stegen na behandeling van de ziekte. Dus in tegenstelling tot onze hypothese lijkt sclerostine geen rol te spelen in het effect van chronisch hypercorticolisme op botformatie. Sclerostine wordt bijna exclusief door osteocyten geproduceerd, cellen waarvan de apoptose door glucocorticosteroïden wordt geïnduceerd. Wij hebben zodoende de mogelijkheid onderzocht dat de verminderde sclerostinewaarden in patiënten met endogeen hypercortisolisme resulteert uit een direct effect van glucocorticoiden op osteocyten. Hiertoe hebben we plasmawaarden gemeten van fibroblast groei factor 23 (FGF23), hetgeen ook uitsluitend door osteocyten wordt geproduceerd. Gelijk sclerostine, namen FGF23 waarden significant toe gedurende biochemische remissie van de ziekten. Dit suggereert dat de lage sclerostinewaarden van patiënten met toegenomen endogene productie van cortisol het resultaat zijn van een verminderd(e)

aantal of functie van osteocyten.

In vitro en *in vivo studies* in diermodellen hebben laten zien dat parathyroïdhormoon (PTH) de expressie van het *SOST* gen remt. In **hoofdstuk 7** testten we de hypothese dat chronisch PTH overschot de hoeveelheid circulerend sclerostine in mensen vermindert. Hiertoe onderzochten we 25 patiënten met toegenomen serum PTH concentraties ten gevolge van primaire hyperparathyreoïdie (PHPT) en 49 patienten genezen van PHPT na succesvolle parathyreoïdectomie. We vonden dat patiënten met PHPT significant lagere serum sclerostinewaarden hadden in vergelijking met patiënten met normale PTH concentraties na parathyroïdectomie. De geobserveerde negatieve correlatie tussen PTH en sclerostinewaarden in deze individuen suggereren dat ook in mensen *SOST* expressie wordt geremd door PTH.

Patiënten met type 2 diabetes mellitus (T2DM) hebben een verhoogd risico op fracturen en thiazolidinedionen (TZDs) verhogen dit risico nog verder. Omdat TZDs de expressie van sclerostine *in vitro* stimuleren, zou een abnormale sclerostineproductie betrokken kunnen zijn in de pathogenese van de toegenomen botfragiliteit in patiënten met T2DM die met TZDs worden behandeld. In **hoofdstuk 8** laten we zien dat mannen met ongecompliceerde T2DM significant hogere sclerostinewaarden hebben in vergelijking met gezonde controles en dat deze waarden toenemen na behandeling met het TZD pioglitazone. In deze patiënten gingen sclerostinewaarden gepaard met een toename van de serummarker van botresorptie CTX. Deze bevindingen suggereren dat een toegenomen sclerostineproductie betrokken kan zijn bij de pathogenese van skeletfragiliteit in patiënten met T2DM en specifiek zouden kunnen bijdragen aan het negatieve effect van TZDs op bot. De behandeling met metformine daarentegen had geen effect op serum sclerostinewaarden en het verminderde de mate van botombouw, waarmee het een gunstiger effect op botmetabolisme heeft dan pioglitazone.

In de voorgaande hoofdstukken hebben we laten zien dat verminderde sclerostinesynthese tot een toegenomen botformatie leidt, maar dat het ook onder sommige condities botresorptie kan stimuleren. In **hoofdstuk 9** onderzochten wij de relatie tussen sclerostine en botombouw in ziekten die gekenmerkt worden door een sterke toegenomen botombouw. Hiertoe hebben wij sclerostinewaarden gemeten in patiënten met de ziekte van Paget, gekenmerkt door met name een verhoogde

botafbraak, in verschillende fasen van ziekte activiteit, en in patiënten met naar het bot gemetastaseerd prostaatcarcinoom, met name gekenmerkt door een toegenomen botformatie, met verschillende mate van ossale metastasering. In vergelijking met gezonde controles waren gemiddelde sclerostinewaarden significant hoger in beide aandoeningen en waren zij positief geassocieerd met de waarden van P1NP, mogelijk ten gevolge van een compensatoire respons tegen het toegenomen aantal osteoblasten in de betrokken skeletlocaties. Daarnaast waren sclerostinewaarden significant geassocieerd met waarden van CTX in patiënten met prostaatkanker, maar niet in patiënten met de ziekte van Paget, hetgeen mogelijk bijdraagt aan de toegenomen botresorptie in patiënten met ossaal gemetastaseerd prostaatcarcinoom.

Concluderende opmerkingen

De beschreven dramatische skeletfenotypen van sclerosteose en de ziekte van Van Buchem illustreren de essentiële rol van sclerostine op de regulatie van botformatie, terwijl onze bevindingen in dragers van deze ziekten aanduiden dat mildere deficiënties in sclerostinesynthese ook geassocieerd zijn met een toegenomen botformatie, maar niet tot complicaties leiden. De gecombineerde resultaten van deze studies ondersteunen het idee voor het gebruik van sclerostineremmers als botopbouwende therapie voor osteoporose. Echter, het uitblijven van verdere progressie van skeletcomplicaties in volwassenheid bij zowel sclerosteose als de ziekte van Van Buchem, en de daling van de waarden van P1NP met de leeftijd in patiënten en dragers, roepen vragen op omtrent de kinetiek van botombouw (tijdelijk of continue) bij lange termijn toediening van sclerostineremmers in oudere individuen.

Gedurende de onderzoeken beschreven in dit proefschrift werden gegevens gepubliceerd die aangeven dat sclerostine niet alleen botformatie remt, maar dat het ook botresorptie kan stimuleren door een RANKL-gemedieerd mechanisme in osteocyten. Sommige van onze resultaten zijn geheel in lijn met deze bevindingen en dragen bij aan een beter begrip van de complexe werking van sclerostine in mensen. In hoeverre metingen van sclerostinewaarden van nut zijn in de klinische praktijk is onzeker, gezien de aanzienlijke overlapping tussen waarden van gezonde controles en

patiënten en de afhankelijkheid van verschillende modulatoren. Aan de andere kant is het ook duidelijk dat sclerostinebepaling als een belangrijk middel kan dienen voor de inschatting van de functie van osteocyten in botmetabolisme in mensen. De huidige en eerder gepubliceerde studies illustreren het belang van osteocyten in de regulatie van botmetabolisme en onderstrepen de rol van sclerostine als een sleutelfactor voor botombouw en botsterkte in mensen.

List of Publications

1. Van Buchem disease: Clinical, biochemical and densitometric features of patients and disease carriers.
   van Lierop A, Hamdy N, van Egmond M, Bakker E, Dikkers F, Papapoulos S.
   J Bone Miner Res. 2012 Oct 16. [Epub ahead of print]

2. Circulating sclerostin levels are decreased in patients with endogenous hypercortisolism and increase after treatment.
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3. Serum sclerostin levels in Paget's disease and prostate cancer with bone metastases with a wide range of bone turnover.
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   Bone. 2012 Jul;51(1):153-7.

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    Bone Miner Res. 2010 Dec;25(12):2796-800.

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Curriculum Vitae

Antoon van Lierop werd op 12 oktober 1981 in Den Haag geboren. Hij slaagde in 2000 *cum laude* voor zijn gymnasium aan het Vrijzinnig Christelijk Lyceum, waarna hij geneeskunde ging studeren aan de Universiteit van Amsterdam. In het kader van zijn wetenschappelijke stage deed hij onderzoek in Vietnam naar de eerste effecten van voedingstransitie, onder begeleiding van dr. P.J. de Vries van het Academisch Medisch Centrum. Na het behalen van zijn artsexamen in september 2008 ging hij als arts-onderzoeker werken op de afdeling Endocrinologie en Metabole ziekten van het Leids Universititair Medisch Centrum, waar hij onder begeleiding van prof. dr. S.E. Papapoulos onderzoek deed naar de rol van het eiwit sclerostine in botmetabolisme. Voor dit onderzoek ontving hij een *Young Investigator Award* van de *European Calcified Tissue Society*, een *Presendent's Poster Award* van de *American Society of Bone and Mineral Research*, en de prijs voor beste voordracht van de Nederlandse Vereniging voor Calcium en Bot. In april 2012 startte hij zijn opleiding interne geneeskunde aan het Academisch Medisch Centrum. Momenteel is hij werkzaam op de afdeling interne geneeskunde van het Rode Kruis Ziekenhuis in Beverwijk.