# Chapter 3

# Serum dickkopf 1 levels in sclerostin deficiency

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# Abstract

Context: Sclerostin and DKK1 are antagonists of the canonical Wnt signaling pathway, both binding to the same LRP5/6 receptor on osteoblasts, thereby inhibiting bone formation. It is not known whether there is an interaction between sclerostin and DKK1.

Objective: We examined whether a lack of sclerostin is compensated by increased DKK1 levels.

Design, setting and patients: We measured DKK1 levels in serum samples of patients and carriers of sclerosteosis (19 patients, 24 carriers) and Van Buchem disease (VBD) (13 patients, 22 carriers), and 25 healthy controls. Sclerosteosis and VBD are caused by deficient sclerostin synthesis and are characterised by increased bone formation and hyperostotic phenotypes.

Main outcome measures: DKK1 levels were compared between patients and carriers, and between patients and healthy controls. We also examined associations between levels of DKK1 and bone turnover markers P1NP and CTX.

Results: We found that DKK1 levels were significantly higher in patients with both sclerosteosis [4.28ng/ml (95%CI: 3.46-5.11ng/ml)] and VBD [5.28 ng/ml (95%CI: 3.84-6.71ng/ml)] compared to levels in carriers of the two diseases [sclerosteosis: 2.03ng/ml (95%CI: 1.78-2.29ng/ml) $p<0.001$, VBD: 3.47ng/ml (95%CI: 2.97-3.97ng/ml) $p=0.017$] and to levels in healthy controls [2.77ng/ml (95%CI: 2.45-3.08ng/ml), $p=0.004$ and $p<0.001$ respectively]. Serum DKK1 levels were positively associated with levels of P1NP and CTX in both disorders.

Conclusions: These results suggest that increased DKK1 levels observed in patients with sclerosteosis and VBD represent an adaptive response to the increased bone formation characterizing these diseases, although these increased levels do not compensate for the lack of sclerostin on bone formation.

# Introduction

Sclerosteosis and Van Buchem disease (VBD) are two rare bone sclerosing dysplasias with closely related phenotypes, characterized by progressive bone overgrowth and very high bone mass [1]. Sclerosteosis is caused by loss-of-function mutations in the *SOST* gene, while patients with VBD lack a regulatory element for the *SOST* gene caused by a 52 kb deletion of genomic DNA 35 kb downstream of the *SOST* gene [1]. In both disorders, these genetic defects lead to impaired synthesis of sclerostin.

Sclerostin is a protein, predominantly synthesized in the skeleton by osteocytes, which decreases bone formation by inhibiting the terminal differentiation of osteoblasts and by promoting their apoptosis. Sclerostin binds to the low density lipoprotein receptor-related protein (LRP) 5/6 receptors and antagonizes the canonical Wnt-signaling pathway in osteoblasts [2]. The binding of sclerostin to LRP5/6 is facilitated by LRP4, a negative regulator of LRP5/6 signaling that augments the inhibitory activity of sclerostin on the Wnt signaling pathway [3]. Dickkopf 1 (DKK1) is a structurally unrelated Wnt antagonist, which similar to sclerostin, blocks the signaling cascade by binding to LRP5/6, a process facilitated by its co-receptor Kremen [4, 5], and inhibits bone formation by acting at different stages of osteoblast development [6]. A direct interaction between sclerostin and DKK1 has not been identified but it has been reported that sclerostin and DKK1 act through a common mechanism involving binding to the first ß-propeller of LRP6 with a conserved amino acid motif present in both proteins, suggesting an interaction between them [7].

Because both sclerostin and DKK1 bind to the same receptors and both proteins inhibit bone formation, we hypothesized that the lack of sclerostin in sclerosteosis and VBD might affect the production of DKK1. In the present study we tested this hypothesis by measuring serum DKK1 levels in patients with sclerosteosis and VBD and in respective heterozygous carriers of these diseases.

# Subjects and methods

## Subjects

Previously collected serum samples from 19 patients with sclerosteosis and 24 carriers of the disease, and from 13 patients with Van Buchem disease (VBD) and 22 carriers of the disease were available for analysis. Detailed clinical characteristics of the subjects have been previously reported [8, 9]. In brief, patients and disease carriers of sclerosteosis were all Afrikaners from South Africa. Patients with sclerosteosis had the characteristic clinical manifestations of the disease, including syndactyly, facial distortion, facial palsy, hearing loss and increased intracranial pressure. None of the disease carriers had any symptoms or complications associated with sclerosteosis. The diagnosis was genetically confirmed in all subjects by the presence of homozygous or heterozygous mutations within the first exon of the *SOST* gene (C69T) in patients and carriers respectively. All patients and disease carriers of VBD were Dutch. Patients with VBD had clinical characteristics and complications similar, but of a milder degree than those of patients with sclerosteosis. In all patients and carriers with VBD the diagnosis was confirmed by presence of homozygous or heterozygous 52kb deletions downstream of the *SOST* gene in patients and carriers respectively.

Informed consent was obtained from all subjects and the study was approved by the Medical Ethical Committee of Leiden University Medical Center.

## Serum biochemistry

Serum samples were measured for sclerostin using the MSD® 96-well MULTI-ARRAY® Human Sclerostin Assay (Meso-Scale Discoveries, Gaithersburg, USA), as previously described [8]. Serum was also measured for calcium adjusted for albumin, phosphate and creatinine using semi automated techniques. Serum procollagen type 1 amino-terminal propeptide (P1NP) and carboxy-terminal cross-linking telopeptide (CTX) levels were determined by the E-170 system (Roche, Basel, Switzerland), 25-hydroxyvitamin D (25OHD) by the LIAISON 25-OH-Vitamin D TOTAL assay (DiaSorine S.A./N.V., Brussels, Belgium) and 1,25-dihydroxyvitamin D (1,25-DHD) concentrations by the LIAISON 1,25-(OH)2 Vitamin D TOTAL assay (DiaSorine S.A./N.V., Brussels, Belgium). Serum DKK1 was measured by

the Quantikine® Human DKK-1 Immunoassay R&D systems (R&D systems Inc., Minneapolis, USA). In our hands the inter-assay and intra-assay precision were 5.8% and 10.4%, respectively. In 25 healthy men and women aged 20 to 63 years, with normal renal function and serum calcium concentrations, and serum P1NP concentration below 65 ng/ml, the mean serum DKK1 level was 2.77 ng/ml (95%CI: 2.45-3.08 ng/ml) range 1.40 to 4.83 ng/ml. These serum DKK1 values are very similar to those reported by the manufacturer of the assay (mean 2.5 ng/ml range 1.4 to 5.2 ng/ml) and by other investigators [10] with this assay (mean 2.4 ng/ml range 1.0 to 3.0 ng/ml). Fibroblast Growth Factor 23 (FGF23) levels were measured using the Kainos Intact Human FGF23 Elisa (Kainos Laboratories Inc., Tokyo, Japan). Intra-assay and inter-assay precisions were 9% and 11%, respectively. In 30 healthy controls with normal renal function, normal calcium and phosphate metabolism and normal serum P1NP levels, mean serum FGF23 was 28.7 pg/ml (95% CI:25.9-31.5 pg/ml).

## Statistical analysis

Differences between patients and carriers of sclerosteosis and VBD were assessed using the Student's t-test. Pearson correlation test was used to assess the relationships between DKK1, biochemical markers of bone turnover and age. The SPSS 17.0 program (SPSS Inc., Chicago, USA) was used for the statistical analysis.

# Results

Characteristics and biochemical measurements of all studied subjects are summarized in Table 1. Subjects were aged between 5 and 82 years. Patients with sclerosteosis were significantly younger than carriers of the disease, although there was no difference in mean age between patients with VBD and carriers.

All studied subjects had normal biochemical indices of mineral metabolism, and normal renal function. As previously reported, serum sclerostin levels were undetectable in patients with sclerosteosis and were very low in patients with VBD. Sclerostin levels were higher in the respective carriers of the two diseases, although still significantly lower than levels measured in healthy subjects [8, 9]. Serum 1,25-DHD concentrations were higher in patients with sclerosteosis compared to carriers of the disease. Serum FGF23 levels were within the normal reference range and not different between patients and carriers of both diseases.

**Table 1.** Group characteristics and biochemical parameters

| | Sclerosteosis | | Van Buchem disease | | |
|---|---|---|---|---|---|
| | **Patients (n=19)** | **Carriers (n=24)** | **Patients (n=13)** | **Carriers (n=22)** | **Reference Range** |
| **Age** | 28.3 ± 15.0 | 41.1 ± 14.5 * | 40.5 ± 21.1 | 38.0 ± 17.3 | |
| **Creatinine (µmol/l)** | 62.2 ± 20.4 | 55.2 ± 12.4 | 77.5 ± 38.5 | 72.2 ± 12.0 | < 120 |
| **Calcium (mmol/l)** | 2.33 ± 0.07 | 2.28 ± 0.11 | 2.25 ± 0.43 | 2.20 ± 0.19 | 2.15 - 2.55 |
| **Phosphate (mmol/l)** | 1.37 ± 0.32 | 1.20 ± 0.32 | 1.12 ± 0.34 | 1.11 ± 0.22 | 0.90 - 1.50 |
| **PTH (pmol/l)** | 5.39 ± 3.20 | 5.03 ± 2.75 | 8.09 ± 5.52 | 6.2 ± 4.17 | 1.5 - 8.0 |
| **1,25-DHD (pmol/l)** | 147.8 ± 55.2 | 92.0 ± 48.6 * | 115.0 ± 56.6 | 117.1 ± 36.7 | 50 - 155 |
| **P1NP (ng/ml)** | 353.8 ± 426.0 | 94.1 ± 122.5 * | 293.1 ± 525.4 | 53.9 ± 24.4 * | < 65 |
| **CTX (pg/ml)** | 397.9 ± 393.8 | 133.5 ± 98.0 * | 855.4 ± 1147.2 | 244.5 ± 136.3 * | < 600 |
| **Sclerostin (pg/ml)** | 0.0 ± 0.0 | 15.9 ± 4.7 * | 8.1 ± 6.2 | 27.2 ± 8.5 * | 14 - 66 § |
| **FGF23 (pg/ml)** | 29.4 ± 16.4 | 33.1 ± 9.1 | 24.8 ± 7.7 | 29.4 ± 9.9 | 13.3 - 44.1 §§ |

Values reported as mean ± standard deviation
* patients vs carriers $p<0.05$
§ defined by mean levels ± 2 SD of 77 healthy controls
§§ defined by mean levels ± 2 SD of 30 healthy controls
PTH=parathyroid hormone
FGF23= fibroblast growth factor
1,25-DHD= 1,25 Dihydroxyvitamine D

Serum DKK1 levels ranged between 1.19 ng/ml and 10.02 ng/ml and children with sclerosteosis or VBD had the highest measured values. Patients with sclerosteosis had significantly higher DKK1 levels than carriers of the disease [4.28 ng/ml (95%CI: 3.46-5.11 ng/ml) vs 2.03 ng/ml (95%CI: 1.78-2.29 ng/ml), p<0.001]. Similarly, patients with VBD had significantly higher serum DKK1 levels [5.28 ng/ml (95%CI: 3.84-6.71 ng/ml)] than carriers of the disease [3.47 ng/ml (95%CI: 2.97-3.97 ng/ml), p=0.017] (Figure 1). These differences between patients with sclerostin deficiency and carriers remained significant after removal of the higher values of the children included in the analysis from the calculations [patients 3.90 ng/ml (95%CI: 3.32-4.48 ng/ml), carriers 2.7 ng/ml (95%CI: 2.39-3.07 ng/ml), p<0.001]. Compared to controls [2.77 ng/ml (95%CI: 2.45-3.08 ng/ml)], patients had significantly higher serum DKK1 values [sclerosteosis p=0.004, VBD p<0.001].

In patients and carriers of both diseases serum DKK1 levels were negatively correlated with age (sclerosteosis: r=-0.64, p<0.001; VBD: r=-0.33, p=0.050). DKK1 levels were positively correlated with serum P1NP levels (sclerosteosis: r=0.78, p<0.001; VBD: r=0.69, p<0.001) and serum CTX levels (sclerosteosis: r=0.74, p<0.001; VBD: r=0.64, p<0.001). These relationships remained significant after adjustment for age.

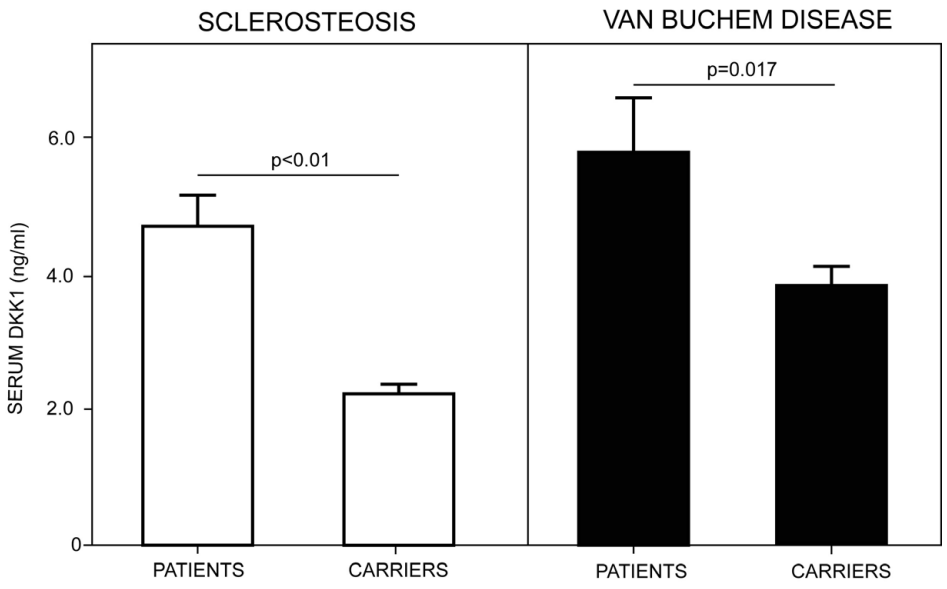


**Figure 1.** Mean serum DKK1 levels in patients and carriers of sclerosteosis (A) and VBD (B). Bars represent SEM.

# Discussion

Our study shows that sclerostin deficiency, as occurs in patients with sclerosteosis or Van Buchem disease, is associated with higher circulating DKK1 levels compared to those of heterozygous carriers of either disease and of healthy subjects. Serum DKK1 concentrations have not been previously reported in sclerostin deficiency, but increased DKK1 expression has been observed in *Sost* knock-out mice (Dr. M. Kneissel, unpublished observations). These findings suggest an interaction between sclerostin and DKK1, two of the known inhibitors of the Wnt-signaling pathway in osteoblasts.

Previously reported measurements of both serum sclerostin and DKK1 in humans have provided variable results not allowing any conclusion to be drawn about a potential functional relationship between the two proteins. For example, serum DKK1 levels were found to be normal in patients with the high bone mass phenotype and high serum sclerostin levels, [11] as was also the case in women with osteoporosis treated with a bisphosphonate [12]. In patients with multiple myeloma and osteolytic bone disease, the levels of both inhibitors were found to be increased [13], whereas DKK1 levels were found to be increased in women with osteoporosis treated with teriparatide, which is associated with a decrease in serum sclerostin levels [14], also consistent with recent findings in patients with primary hyperparathyroidism [15]. Conversely, treatment with denosumab was associated with decreases in serum DKK1 and increases in serum sclerostin levels [16].

The increased serum DKK1 levels in patients with sclerostin deficiency we found in the present study may be related to the higher rate of bone formation and turnover observed in these patients, as illustrated by the positive correlation between DKK1 and the bone turnover markers P1NP, and CTX. Alternatively or in addition to this potential explanation, the higher serum DKK1 levels found in sclerostin deficiency may be an adaptive response to the lack of sclerostin, in an attempt to protect the skeleton from unrestrained bone formation. This notion is supported by the finding of the highest DKK1 values in children in whom the effect of sclerostin deficiency is most pronounced [8, 9]. Previous studies have shown no association between DKK1 values and age in either healthy adults [17-19] or children (Dr. P. Dimitri, personal communication). In sclerosteosis and VBD, bone overgrowth is progressive mainly during childhood, and both diseases stabilize during adulthood, as shown by a decline in serum levels of PINP with age [8, 9]. The finding of the

highest DKK1 levels in children is, therefore, in keeping with the natural course of the two sclerosing disorders and with the potential compensatory role of DKK1 for the underlying sclerostin deficiency. The hyperostotic phenotypes of sclerosteosis and VBD indicate, however, that the increased DKK1 synthesis is not able to fully compensate for the lack of sclerostin in these patients. Whereas both Wnt antagonists act by binding to the same LRP5/6 receptor, they have different mechanisms of action and expression pattern (sclerostin is expressed in bone by osteocytes while DKK1 by osteoblasts and osteocytes and outside bone) and they act on osteoblasts at different developmental stages. This might explain therefore why the functions of these two proteins are not fully compensatory, although sclerostin and DKK1 are both antagonists of the Wnt signaling pathway in osteoblasts.

Despite the profound changes in bone metabolism observed in patients with sclerostin deficiency, mineral metabolism is not generally affected in these patients. A recent study in *Sost* knock-out mice reported increased serum concentrations of 1,25-DHD and decreased levels of FGF-23 in these mice, suggesting that sclerostin deficiency may also affect mineral metabolism [20]. In contrast to patients with VBD, in whom there was no difference in 1,25-DHD levels between patients and carriers, we found higher serum 1,25-DHD concentrations in patients with sclerosteosis compared to carriers of the disease. Serum 1,25-DHD concentrations bore, however, no relationship to serum FGF-23 levels, which were within the normal range in all subjects whether patient or carrier, with no difference between patients and carriers of both diseases or healthy controls. Our results in humans do not, therefore, support the findings from the animal study.

In conclusion, we hypothesize that the higher levels of DKK1 we found in patients with sclerosteosis and Van Buchem disease and their carriers are a compensatory response to the prevalent sclerostin deficiency found in both diseases. This response is however insufficient to protect against the excessive bone formation characteristic of these disorders.

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