Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2021 Dec 13; 9(B):1664-1671.
https://doi.org/10.3889/oamjms.2021.7578
eISSN: 1857-9655
Category: *B - Clinical Sciences*
Section: *Nephrology*

OPEN ACCESS

The Relationship between Serum Sclerostin Levels and Bone Mineral Disorders and Vascular Calcification in Hemodialysis Patients

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Edited by: Ksenija Bogoeva-Kostovska
Citation: Abdallah E, Sherif N, Mosbah O, Metwally A, Abd ElAzim I, Mahmoud O, Farouk M, Mamdouh S, El-Shishtawy S. The Relationship between Serum Sclerostin Levels and Bone Mineral Disorders and Vascular Calcification in Hemodialysis Patients. Open-Access Maced J Med Sci. 2021 Dec 13; 9(B):1664-1671. https://doi.org/10.3889/oamjms.2021.7578
Keywords: Bone mineral densitometry; End-stage renal disease; Hemodialysis; Vascular calcification
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Received: 12-Oct-2021
Revised: 06-Dec-2021
Accepted: 09-Dec-2021

Funding: This research did not receive any financial support
Competing Interest: The authors have declared that no competing interest exists

Abstract

BACKGROUND: Sclerostin is produced by osteocytes and has been shown to down-regulate the synthesis of many markers of bone formation by osteogenic cells.

AIM: The aim of this study is to investigate the relationship between serum sclerostin levels and bone mineral disorders and vascular calcification in hemodialysis (HD) patients.

METHODS: This is a cross-sectional study of 70 patients with end-stage renal disease (ESRD) on regular HD for at least 6 months, Theodor Bilharz Research Institute, Giza, Egypt. Twenty-five subjects who matched the ages, genders, and demographics of the study patients were included as a control group. All patients and control groups included in the study underwent a full through history and clinical examination. Serum calcium, phosphorus, alkaline phosphatase (ALP), and intact PTH (iPTH) levels were measured. Serum sclerostin was measured by an ELISA. Bone mineral densitometry (BMD) measurements (g/cm^2) were determined by dual-energy X-ray absorptiometry. CT scan was done to detect the presence or absence of vascular calcification and transthoracic echocardiogram to detect the presence or absence of valvular calcification.

RESULTS: The mean seumscleostin levels was a statistically significant high in the HD patients when compared with the control group (156.8 ± 121.4 vs. 29.38 ± 0.84, p = 0.0001) and statistically significant high mean ALP in the HD patients when compared with the control group (147.2 ± 94.3 vs. 38.8 ± 23.4, p = 0.0001). The mean BMD was statistically significant low in the HD patients when compared with the controls (0.839 ± 0.086 g/m^{2} vs. 1.306 ± 0.153 g/m^2, p = 0.0001). The mean seumscleostin levels were statistically significant high in the HD patients with vascular and valvular calcification when compared with HD patients without calcification. Using spearman correlation coefficient analysis, there was statistically significant negative correlations between serum sclerostin levels and iPTH (r = –0.362, p = 0.0021), ALP (r = –0.301, p = 0.0114), and BMD (r = –0.469, p = 0.0278), and there was a statistically significant positive correlation between serum sclerostin levels and phosphate (r = 0.5829, p = 0.0001). Independent predictors of BMD in HD patients were determined using multi-variate regression analysis. Sclerostin levels, iPTH, ALP, and age were found to be independent predictors of BMD.

CONCLUSION: High sclerostin levels in patients with ESRD on HD were associated with high risk of vascular and valvular calcification and were independent predictors of low BMD in such population.

Introduction

Sclerostin is a 190-residue secreted glycoprotein that is predicted to contain a cysteine-knot motif and is a member of the DAN/Cerberus protein family [1]. Sclerostin is produced by osteocytes and has been shown to down-regulate the synthesis of many markers of bone formation by osteogenic cells [2], [3], [4], thereby indicating the importance of sclerostin in the regulation of bone formation.

Chronic kidney disease-mineral bone disorder (CKD-MBD) is defined by abnormalities in mineral and hormone metabolism that, decrease bone health and increase soft tissue calcification [5], [6]. Several studies have shown that these changes are associated with increased fracture rates and cardiovascular complications, including decreased vascular wall elasticity, vascular calcification, and left ventricular hypertrophy [7], [8].

Wnt/β-catenin signaling plays a key role in various biological processes, including cell proliferation, cell migration, and differentiation [9]. Wnt ligands bind to cell surface receptor complexes that are comprised of Frizzled and low-density lipoprotein receptor-related protein (LRP) family members [9], [10]. Sclerostin is a soluble Wnt inhibitor, Wnt ligands are blocked from binding the LRP-5/6-Frizzled receptor complex. Since Wnt signaling encompasses vascular development and endothelial cell specification as well as regulation of bone modeling and remodeling, it appears prototypic for the crosstalk within the bone-vascular axis [11], [12], [13].

Taken together, the above findings are consistent with a hypothesis that increased serum Wnt antagonist levels induce low bone turnover, thereby indirectly increasing the propensity for vascular calcification. In suspect of this hypothesis, the serum sclerostin levels of hemodialysis (HD) patients have been found to be positively associated with coronary and aortic valve calcifications, and higher expression of sclerostin has been observed close to calcified areas in explanted aortic valves from dialysis patients beyond bone mineralization [14], [15]. Moreover, the results of a recent study of prevalent HD patients indicated that serum sclerostin is an independent predictor of mortality [16]. The aim of the present study was to investigate the association of circulating concentrations of sclerostin with MBD and vascular calcification in hemodialysis patients.

Methods

Study population

This is a cross-sectional study of 70 patients with end-stage renal disease (ESRD) on regular HD for at least 6 months in the Nephrology department, Theodor Bilharz Research Institute, Cairo, Egypt. All patients received three, 4-h dialysis sessions/week. Patients with congestive heart failure, malignancy and sepsis, and/or liver, autoimmune, and chronic inflammatory diseases were excluded from the study.

Twenty-five subjects who matched the ages, genders, and demographics of the study patients were included as a control group.

All patients and control groups included in the study underwent a full through history and clinical examination. Demographic and clinical characteristics, including age, gender, body mass index (BMI), blood pressure (BP), duration of dialysis, and etiology of ESRD, were recorded.

Ninteen HD patients received quadriple antihypertensive medications (angiotensin converting enzyme inhibitors [ACEI] or angiotensin receptor blockers (ARBs), calcium channel blocker [amlodipine], beta blocker [atenolol] and vasodilators [hydralazine]), 17 received triple antihypertensive medications (ACEI or ARBs, amlodipine and atenolol), 14 received two antihypertensive medications (ACEI or ARBs and amlodipine), 13 received monotherapy (ACEI or ARBs or amlodipine or hydralazine) and 7 HD patients did not receive antihypertensive medications. Patients were prescribed treatments including $CaCO_3$ (21 patients), sevelamer-HCl (18 patients), calcitriol (46 patients), cinacalcet (16 patients), antiplatelet agents (34 patients), warfarin (3 patients), and erythropoietin (43 patients).

Laboratory parameters

Blood sample collection

Fasting blood samples were collected in tubes from the patients and control groups after proper disinfection. The tubes were centrifuged at 4000 rpm (10 min) to obtain plasma and serum. The plasma and serum samples were kept at −80°C until analysis of sclerostin. Routine examinations included complete blood picture using tripotassium EDTA-based anticoagulated blood samples (Sysmex K-1000 auto analyzer, Block Scientific, USA) within 30 min of sampling [17], kidney function tests (serum creatinine, urea, sodium and potassium, and uric acid), random blood sugar, total cholesterol, low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), and triglycerides (TGs) using standard methods. Intact PTH (iPTH) level was measured by a radioimmunometric assay (Scantibodies, Santee, CA): normal range is 14–66 pg/ml; intra- and inter-assay coefficients of variation are <5 and <7%, respectively. Blood chemistry measurements for calcium, phosphorus alkaline phosphatase (ALP) were done using automated techniques. C-reactive protein (C-RP) was measured using a BN2 model nephelometer (Dade-Behring, Germany) [18].

Serum bone alkaline phosphatase (BAP) was measured, as a marker of bone formation, using an enzyme immunoassay kit (Alkphase-B; Metra Biosystem) [19]. The assay detected 0.7–140 U/L of BAP. The intra- and interassay coefficients of variation were 2.3 and 3.1%, respectively [20].

Measurement of serum sclerostin

Sclerostin was measured by an ELISA. Briefly, microtiter plates (MaxiSorp; Nunc-Thermo Fisher Scientific, Waltham, MA) were coated with 100 μl of monoclonal anti-sclerostin antibody (MAB1406; R&D Systems, Minneapolis, MN) at a concentration of 2 μg/ml in carbonate buffer (pH 9.6) and were incubated at 4°C overnight. Plates were washed with PBS containing 0.05% Tween 20 (Sigma-Aldrich, Vienna, Austria) and blocked with PBS containing 0.05% Tween and 1% human serum albumin (Sigma-Aldrich). Fifty microliters of serum was loaded per well, incubated overnight at 4°C, washed, and incubated for 1 h at 37°C, followed by incubation at 4°C for 1 h with a biotinylated polyclonal anti-sclerostin antibody (BAF 1406; R&D Systems) diluted to a concentration of 0.5 μg/ml in dilution buffer. Wells were washed with PBS/Tween, followed by the addition of 100 μl of a 1:20,000 dilution of streptavidin–horseradish peroxidase (Endogen-Thermo Fisher Scientific, Waltham, MA). Color development was achieved with the tetramethylbenzidine substrate system (Chemicon-Millipore, Billerica, MA). Serial dilutions of recombinant human sclerostin (1406-ST; R&D Systems) were used to establish a standard curve. Normal values in 44 healthy volunteer's age 19–76 years are between 131 and 1156 pg/ml; intra- and inter-assay coefficients of variation are 7.5 and 6.3%, respectively [21].

Bone mineral densitometry (BMD) measurements

BMD measurements (g/cm^2) were determined for the anteroposterior lumbar spine (L1–L4) and mean of proximal right and left femur (total and subregions) by dual-energy X-ray absorptiometry, using LUNAR Prodigy Model (Lunar Corp., Madison, WI, USA) according to standard protocol. Quality-control procedures were carried out in accordance with the manufacturer's recommendations as described previously [22]. BMD values were classified according to the WHO criteria: a T-score between _1 and _2.5 is indicative of osteopenia, whereas a T-score of _2.5 and below reflects osteoporosis, and a T-score of _1.0 and above is considered normal [23].

MDCT scan to detect vascular calcification and transthoracic echocardiogram

Vascular calcification was evaluated using MDCT scan to detect the presence or absence of vascular calcification along the whole course of aorta on the thorax, abdomen if present; and transthoracic echocardiogram to detect the presence or absence of valvular calcification.

Statistical analysis

Data were presented as mean ± SD. Or number of cases and percentages. Comparisons between variables in the study groups were performed using unpaired two-tailed Student's t-tests ((MedCalc Statistical Software). Correlation between sclerostin levels and various variables was done using Spearman correlation coefficient analysis (MedCalc Statistical Software). Independent predictors of BMD in HD patients were determined using multi-variate regression analysis (MedCalc Statistical Software). p < 0.05 was considered statistically significant.

Results

The baseline demographic and clinical characteristics of the study population are shown in Table 1. The mean age of the patients was 54.9 ± 14.46 years, and the mean dialysis duration was 65.6 ± 36.8 months. The main causes of ESRD were diabetes mellitus (DM), hypertension, glomerulonephritis, obstructive uropathy, and adult polycystic kidney disease.

Table 1: Demographic and clinical characteristics of hemodialysis patients and control group

Variables	HD patients (n = 70)	Control (n = 25)	*p*-value
Age (years)	54.9 ± 14.46	50.6 ± 15.20	0.3207
Gender (M/F)	42/28 (59.7%/41.3%)	16/9 (62.5%/37.5%)	0.9985
BMI (kg/m^2)	25.3 ± 3.8	26.1 ± 2.90	0.4522
SBP (mm/Hg)	151.7 ± 17.8	117.8 ± 8.40	0.0001
DBP (mm/Hg)	95.8 ± 11.8	78.7 ± 11.80	0.0001
Mean BP (mm/Hg)	114.4 ± 13.991	7 ± 10.60	0.0001
Duration of dialysis (months)	65.6 ± 36.8		
Aetiology of ESRD			
Diabetes mellitus	27 (38.9%)		
Hypertension	23 (31.9%)		
Glomerulonephritis	12 (17%)		
Obstructive uropathy	4 (5.7%)		
APKD	2 (2.3%)		
Unknown	2 (3.4%)		

HD: Hemodialysis, BMI: Body mass index, Mean BP: Mean blood pressure, ESRD: End-stage renal disease, APKD: Adult polycystic kidney disease

The laboratory parameters of the study population are shown in Table 2. When compared with the control group, HD patients had higher levels of serum creatinine, phosphorus, and PTH. There was a statistically significant low mean value of 25-OH Vitamin D$_3$ in HD patients when compared with the controls (44.56 ± 17.35 vs. 55.76 ± 23.75 respectively, p =0.0141).

Table 2: Laboratory parameters of hemodialysis patients and control group

Variables	HD (n = 70)	Control (n = 25)	*p*-value
S. creatinine (mg/dl)	8.3 ± 1.8	0.85 ± 0.60.	0.0001
S. calcium (mg/dl)	8.8 ± 0.9	9.3 ± 0.60	0.1046
S. phosphorus (mg/dl)	5.7 ± 1.1	3.63 ± 0.550	0.0001
S. PTH (pg/ml)	269.87 ± 63.76	34.7 ± 4.680	0.0001
T.cholesterol (mg/dl)	154.5 ± 17.8	119.7 ± 11.90	0.0001
LDL-C (mg/dl)	126.1 ± 30.2	98.8 ± 24.40.	0.0037
HDL-C (mg/dl)	37.12 ± 8.1	48.36 ± 7.210	0.0001
TGs (mg/dl)	119.2 ± 23.2	88.9 ± 19.10	0.0001
Hb (g/dl)	11.4 ± 1.5	14.5 ± 1.20	0.0001
S. glucose (mg/dl)	127.8 ± 25.7	118.5 ± 20.60	0.0898
C-RP (mg/l)	18.1 ± 4.6	0.74 ± 0.50	0.0001
S. albumin (g/dl)	3.7 ± 0.6	4.3 ± 0.40	0.0001
Serum uric acid (mg/dl)	7.2 ± 0.5	5.6 ± 0.80	0.0001
S. BAP (U/L)	42.7 ± 21	219.6 ± 7.3	0.0001
Total ALP	147.2 ± 94	338.8 ± 23.40	0.0001
S.sclerostin (pmol/L)	156.8 ± 121	429.38 ± 0.840	0.0001
25-OH Vit. D$_3$ (ng/ml)	44.56 ± 17.3555	76 ± 23.750	0.0141
BMD (g/cm^2)			
Nek femur	0.839 ± 0.086	1.306 ± 0.1530	0.0001

PTH: Parathyroid hormone, T.cholesterol: Total cholesterol, LDL-C: Low density lipoprotein-cholesterol, HDL-C: High density lipoprotein-cholesterol, TGs: Triglycerides, Hb: Hemoglobin, C-RP: C-reactive protein, PON-1

The mean serum scleostin levels was a statistically significant high in the HD patients when compared with the control group (156.8 ± 121.4 vs. 29.38 ± 0.84, p = 0.0001) and statistically significant high mean ALP in the HD patients when compared with the control group (147.2 ± 94.3 vs. 38.8 ± 23.4, p = 0.0001). The mean BMD was statistically

Table 3: Multi-variate linear regression analysis of the predictive factors of BMD

Variables	β	*p*-value
Parathyroid hormone	-0.31	0.023
Duration of hemodialysis	-0.22	0.095
Sclerostin	-0.29	0.031
Age	0.32	0.021
BMI	0.21	0.096
ALP	-0.34	0.019

BMD: Bone mineral densitometry, BMI: Body mass index, ALP: Alkaline phosphatase significant low in the HD patients when compared with the controls (0.839 ± 0.086 g/m^2 $^{vs.}$ 1.306 ± 0.153 g/m^2 respectively, p = 0.0001).

The mean seumscleostin levels were statistically significant high in the HD patients with vascular and valvular calcification when compared with HD patients without calcification.

The mean values of serum sclerostin were higher in male HD patients than female HD patients and high in patients with type 11 DM when compared with non-diabetic patients.

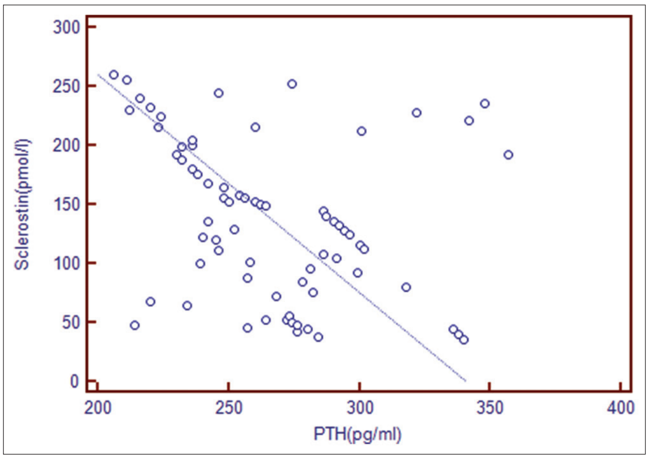

Figure 1: Correlation between serum sclerostin and parathyroid hormone levels (r = –0.362, 95% CI for r-0.550—0.139, p = 0.0021)

Spearman correlation coefficient analysis of serum sclerostin levels with MBD markers in HD patients demonstrated statistically significant negative correlations between serum sclerostin levels and iPTH (r = –0.362, 95% CI for r –0.550—0.139, p = 0.0021; Figure 1), ALP (r = –0.301, 95% CI for r –0.500—0.0706, p = 0.0114; Figure 2), and BMD (r = –0.469, 95% CI for r –0.508—0.0298, p = 0.0278; Figure 3); there was a statistically significant positive correlation between serum sclerostin levels and phosphate and age (r = 0.5829, 95% CI for r 0.4031–0.7193, p = 0.0001; Figure 4).

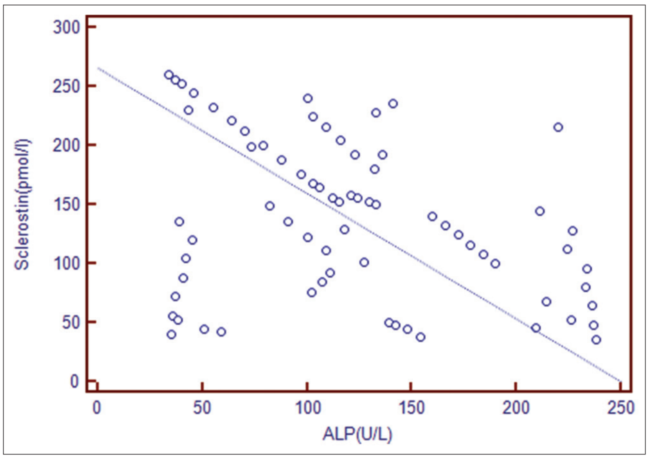

Figure 2: Correlation between serum sclerostin and alkaline phosphatase levels (r = –0.301, 95% CI for r –0.500—0.0706, p = 0.0114)

There was no a significant correlation between serum sclerostin levels and serum calcium, 25-OH

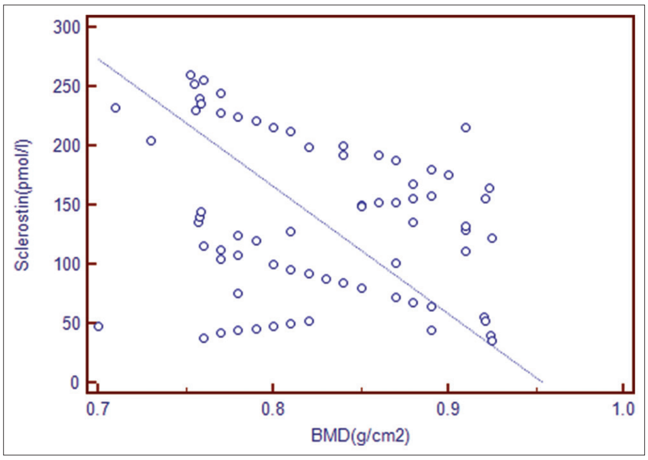

Figure 3: Correlation between serum sclerostin levels and bone mineral densitometry (r = –0.469, 95% CI for r –0.508—0.0298, p = .0278)

Vitamin D_3, dialysis duration, BMI, uric acid, serum albumin, C-RP, hemoglobin, LDL- and HDL-cholesterol, TGs and systolic and diastolic BP.

Independent predictors of BMD in HD patients were determined using multi-variate regression analysis (Table 3). Sclerostin levels, iPTH, ALP, and age were found to be independent predictors of BMD.

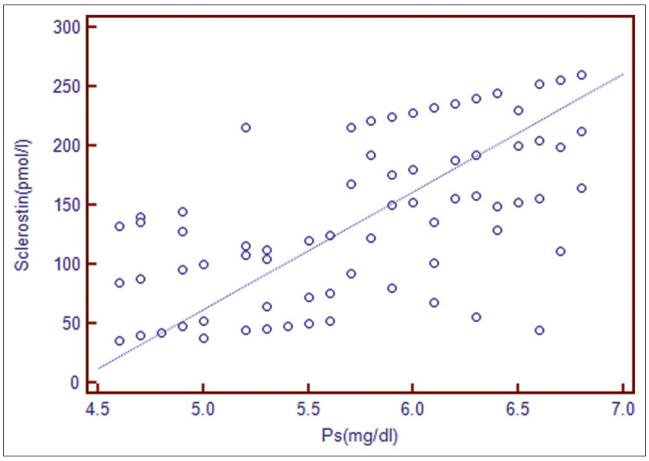

Figure 4: Correlation between serum sclerostin and phosphate levels (r = 0.5829, 95% CI for r 0.4031–0.7193, p = 0.0001)

Discussion

The present study demonstrated that mean values of serum sclerostin levels were significantly high in HD patients when compared with the control group and that serum sclerostin levels correlated negatively with iPTH, BAP and BMD and positively with Ps.

Sclerostin is mainly secreted by osteocytes, and it decreases bone formation by inhibiting the terminal differentiation of osteoblasts and promoting their apoptosis. Sclerostin blocks Wnt signaling pathway in osteoblasts by binding to LRP-5/6 receptors [13]. Wnt signaling and bone morphogenetic protein are involved in osteoblastogenesis and bone formation [24]. Sclerostin is also expressed in several non-skeletal tissues, especially in the vasculature, but whether this is a cause or a consequence of vascular calcification is yet to be determined [25].

CKD-MBD is a syndrome of bone disorders and is associated with disturbances in calcium and phosphorus homeostasis in association with hyperparathyroidism [26]. PTH is known to bind directly to cells of the osteoblast/osteocyte lineage and promote increased RANKL expression, which leads to osteoclast activation.

In our work, mean value of serum sclerostin levels was significantly high in HD patients when compared with the control group. The molecular size of sclerostin is approximately 22.5 kDa, and filtered through glomeruli and reabsorbed by renal tubular cells in a normal kidney. A decrease in glomerular filtration rate (GFR) and/or increased sclerostin production by osteocytes in CKD patients may lead to high serum sclerostin due to accumulation of sclerostin in the serum. There is a controversy for the mechanism involved in increased serum sclerostin levels in CKD patients. For example, Cejka *et al*. reported that renal elimination of sclerostin increased regardless decreased renal function and urinary sclerostin excretion increased with declining GFR [27]. Furthermore, increased extraskeletal production of sclerostin may be one of the causes of its high serum levels. For example, Roforth *et al*. reported that bone mRNA levels did not increase in older people regardless of their high serum sclerostin levels [28]. Circulating sclerostin levels have been found to be increased in several cohorts of CKD patients. Cejka *et al*. were the first to report finding increased serum sclerostin levels in a cross-sectional study of dialysis patients [29], and their finding has been validated by other studies in ESRD patients [25], [21], [30], [31], [32], [33]. Pelletier *et al*. reported that higher serum sclerostin levels were starting at CKD Stage 3 [31]. However, the degree to which serum sclerostin levels reflect changes in expression versus accumulation in individuals with impaired renal function is not fully understood. A previous study examining the local expression of sclerostin across stages of CKD revealed that highest osteocyte expression occurred at initial stages of the disease [34]. Although this study examined the number of sclerostin-positive osteocytes rather than absolute protein levels, the resulting data suggest that sclerostin accumulation in the serum is at least partially due to increased osteocyte production. Moreover, the rapid restoration of serum sclerostin to the normal range post-transplant suggests that decreased renal clearance may also be responsible for accumulation at least in late stages [35].

The present study demonstrated that serum sclerostin levels correlated negatively with iPTH and correlated positively with phosphate. Our results are consistent with many previous study. Delanaye *et al*. reported finding that plasma sclerostin levels in HD patients were positively associated with their phosphate levels and negatively associated with their PTH levels [36]. A recent study demonstrated an increased serum sclerostin levels and that serum sclerostin were closely associated with serum phosphate and FGF23 levels and treatment with Vitamin D in HD patients with low serum PTH levels [37]. Furthermore, several previous studies showed a significant and negative correlation between serum sclerostin levels and serum iPTH in non-CKD [38], [39] and HD patients [29], [21].

Our study showed that there was a significant negative correlation between serum sclerostin levels and BMD. These findings are consistent with the hypothesis that, as would be expected of a negative regulator of bone formation, higher serum sclerostin levels promote low bone turnover, which leads to loss of bone mass over time. Our results are consistent with many previous studies. A cross-sectional study of 60 dialysis patients showed that their serum sclerostin levels were inversely correlated with the patients' bone formation rates [32]. A subsequent prospective study of 81 dialysis patients found that higher sclerostin serum levels predicted greater loss of bone mass over a 1-year period [33]. However, in contrary to our results, Cejka *et al*. [29] observed positive correlations of serum sclerostin with BMD and considering that sclerostin is an inhibitor of bone formation, the observed results were unexpected. Whether its increase in dialysis patients has direct pathogenetic relevance or is only a secondary phenomenon remains to be seen.

In this work, we founded that the mean values of serum sclerostin were high in HD patients with vascular and valvular calcification when compared with HD patients without calcification. Recent study findings indicate that sclerostin is involved in vascular disease. An in vitro and rodent study by Zhu *et al.* could show that sclerostin is upregulated in experimental models of vascular calcification [40]. We extend these findings for a potential linkage between serum sclerostin levels and vascular and valvular calcification in humans with ESRD. Our results are consistent with recent human study results indicating an association of sclerostin expression with non-uremic aortic valve calcification [41] and Brandenburg *et al*. [14] found a strong association of sclerostin with calcifying aortic heart valve disease in hemodialysis patients and sclerostin is locally produced in aortic valve tissue adjacent to areas of calcification. Therefore, sclerostin appears to be a promising future research target in CKD-MBD offering potential therapeutic perspectives [42], [43].

In the present study, serum sclerostin levels were high in male HD patients when compared with female HD patients and were significantly correlated with age. Furthermore, serum sclerostin levels were high in diabetic HD patients when compared with non-diabetic HD patients. Our results are consistent with many previous studies. Serum sclerostin levels were significantly correlated with age and were higher in male than female patients with Stage 3b and 4 CKD [44]. Larger bone mass in males, hormonal effect (a role of estrogen in reducing sclerostin levels), skeletal remodeling and imbalances in vascular remodeling with aging in males might be responsible for the observed differences [45], [46], [47]. In non-CKD patients, serum sclerostin levels have been reported to be higher in males, in patients with higher age, and in patients with Type 2 diabetes [39], [45], [46], [48], [49]. In another study of HD patients, serum sclerostin was also higher in males than females. However, age was not associated significantly with serum sclerostin levels, and serum sclerostin levels were not different between patients with and without diabetes [32].

Conclusion

This study demonstrated significant high serum sclerostin levels in HD patients and high serum sclerostin levels were associated with high risk of vascular and valvular calcification and were independent predictors of low BMD in such population.

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