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ORIGINAL ARTICLE

Sclerostin and chronic kidney disease: the assay impacts what we (thought to) know

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ABSTRACT

Background. Sclerostin, a 22-kDa protein secreted by osteocytes, acts as a potent inhibitor of osteoblast activity. In chronic kidney disease (CKD), sclerostin is a putative driver of the bone–vascular axis. However, large discrepancies between sclerostin assays have been described.

Methods. We compared four different assays [Biomedica (BM), TecoMedical (TE), R&D (RD) and MesoScaleDiscovery (MSD)] in an analytical study and addressed the question whether bioassay choice affects the correlation between circulating sclerostin and clinical and biochemical determinants. Circulating sclerostin levels were determined in 39 prevalent dialysis patients and 82 non-dialysis patients referred for glomerular filtration rate measurement.

Results. In the 82 non-dialysis patients, we observed large differences in median (interquartile range) sclerostin concentrations (in pg/mL): BM, 984 [interquartile range (IQR) 648]; TE, 629 (IQR 237); RD, 154 (IQR 84) and MSD, 36 (IQR 19). The concordance correlation coefficient between assays was poor (0.1–0.44). The same discrepancies were observed in dialysis patients. A significant negative rank correlation was found between glomerular filtration rate and sclerostin measured by BM and TE but not by MSD and RD. Associations between sclerostin and age, gender, weight or parathormone were also different according to the assay considered.

Conclusions. Clinical inference relating sclerostin levels found in the general, CKD and dialysis populations is largely influenced by the assay used to measure this biomarker.

Keywords: biomarkers, chronic hemodialysis, chronic kidney disease, PTH, sclerostin

INTRODUCTION

Sclerostin, a 22-kDa protein secreted by osteocytes and chondrocytes, was originally described in humans with mutation of its producing gene (*SOST*), leading to a phenotype characterized by a high bone mass (sclerosteosis) [1]. Further research suggested that sclerostin acts as a potent inhibitor of osteoblast activity and thus bone formation. Recent evidence points to sclerostin as the long missing link between osteocytes as mechanoreceptors and bone formation. Increases in loading-engendered strains down-regulate osteocyte sclerostin expression, whereas reduced strains, as in disuse, are associated with increased sclerostin production and bone loss [2, 3]. The discovery of this pathway opened new avenues for the treatment of osteoporosis [4, 5]. Sclerostin has also been extensively studied in the context of chronic kidney disease (CKD), especially as a putative driver of the bone–vascular axis [6–10]. Numerous correlates have been identified, including age [11–14], glomerular filtration rate (GFR) [11, 15–17] and parathyroid hormone (PTH) [18–20]. Many investigators have reported associations between circulating sclerostin levels and indices of bone and vascular health and mortality [10–12, 14, 15, 19–32]. The strength and even the direction of these associations showed important differences between studies. Besides case mix, analytical issues may have contributed to the discrepant findings [33]. In the present study we compare the performance of four commercially available sclerostin assays and thereby focus on the impact of the assay on the relationship between circulating sclerostin level and putative and established clinical and biochemical determinants [8].

MATERIALS AND METHODS

Population

Circulating sclerostin levels were determined in 39 prevalent dialysis patients and in 82 non-dialysis patients referred for GFR measurement using iohexol plasma clearance, as previously described [34, 35]. In the dialysis cohort, 21 patients were treated by haemodiafiltration (HDF), all in post-dilution mode (with a minimal ultrafiltration rate ∼100 mL/min) using polysulfone membranes, while 18 patients were treated with conventional

haemodialysis (HD) using polysulfone membranes. The protocol was approved by the Ethics Committee (B7072014220701 for CKD and healthy subjects and B707201215885 for dialysis patients) and all patients signed informed consent.

Sample collection, assays and calculations

Blood samples were collected between 2013 and 2016. In non-dialysis patients, samples were obtained at the time of the GFR determination. In dialysis patients, blood samples were collected before and after the mid-week dialysis session. Samples were immediately centrifuged and kept frozen at −80°C until determination.

Serum calcium, phosphorus and albumin (Roche Cobas, Mannheim, Germany), PTH and 25-OH vitamin D (DiaSorin Liaison, Saluggia, Italy) were determined by standard techniques in non-dialysis patients. Serum sclerostin measurements were performed in batch between December 2015 and January 2016 (Department of Clinical Chemistry, University of Liège, accredited for the ISO 15189 Standard), using four different immunoassays: Biomedica Sclerostin (Biomedica Gruppe, Wien, Austria; BM), lot Y154; TecoMedical Sclerostin HS, (TECOmedical, Sissach, Switzerland; TE), lot 046435; R&D Human SOST (R&D Systems, Minneapolis, MN, USA; RD), lot 338535; and MesoScaleDiscovery 96-well MultiArray Human Sclerostin (MesoScaleDiscovery, Gaithersburg, MD, USA; MSD), lot K0050700. Assay specificities are summarized in Table 1 [33]. Interassay coefficients of variation were <10% for all assays.

Results after dialysis were corrected for total extracellular volume change by dividing raw values by $(1+(\Delta BW/0.2\times BW))$, where ΔBW and BW are the loss of bodyweight during dialysis and post-dialysis bodyweight, respectively [36].

Statistics

Data are expressed as mean ± SD when distribution was normal and as median with interquartile range (IQR) when not. Normality was assessed by the Shapiro–Wilk test. Correlation between assays was evaluated by Spearman rank correlation (ρ) and agreement with Lin's concordance correlation coefficient (CCC) and Bland and Altman analysis. Correlation between sclerostin measurements from each assay and GFR determinations were evaluated by Spearman rank correlation. We performed analysis of variance (ANOVA), followed by Tukey's honest significant difference (HSD) test (for pairwise comparisons), which controls the Type I error rate, to enable the comparison of sclerostin test results between the healthy, CKD and dialysis subgroups. Backward and forward multiple regression analysis using $\alpha = 0.05$ as the entry criterion was used to study the potential linear relationship between sclerostin and clinical or biological data in 82 patients. For the comparison of concentration results between assays before and after dialysis, we considered repeated measures ANOVA to compare tests among each other, followed by Tukey's post-test (for pairwise comparisons). The Wilcoxon rank sum test was used to test the equality of mean relative change between HD and HDF sessions. All statistical analyses were conducted using Medcalc (Mariakerke, Belgium) and SAS 9.3 (SAS Institute, Cary, NC, USA).

RESULTS

Demographics and biochemistry

Relevant demographics and biochemistry data are summarized in Table 2.

Table 1. Comparison of the commercially available sclerostin assays used in the study, according to the manufacturers

Manufacturer	Assay name	Assay type	Primary/ secondary antibody	Intra- and interassay CV	Range/ sensitivity (pg/mL)	Expected range	Standard source
Biomedica	Sclerostin ELISA	ELISA	Polyclonal/ monoclonal	<7% <10%	0–5400 LOD: 72 LLOQ: <170	Median of 411 healthy individuals: 543 pg/mL	Not defined
TECOmedical	TECOmedical Sclerostin HS EIA	ELISA	Polyclonal/ monoclonal	<5% <5%	50–3000 LOD: 9 LLOQ: 58 LOQ: 350	Pre-menopause (n = 24): 450 ± 150 pg/mL Post-menopause (n = 20): 510 ± 140 pg/mL Men (n = 11): 590 ± 130 pg/mL	Not defined
MesoScaleDiscovery	Human Sclerostin	EIA	Polyclonal/ polyclonal	Not available in the IFU. Our intra-assay CV ranged from 5 to 12% and interassay CV from 8 to 17%	4–10 000 LOD: 4	Not provided by the manufacturer	Recombinant human protein expressed in a mouse myeloma cell line
R&D systems	Quantikine ELISA human SOST immunoassay	ELISA	Monoclonal/ polyclonal	<3% <11%	0–2000 LOD: 1.7	Serum (n = 35): 174 ± 61 pg/mL; range: 67.0–300 pg/mL	A highly purified NS0-expressed recombinant human SOS manufactured at R&D

CV, coefficient of variation; ECL, electrochemiluminescence assay; EIA, enzyme immuno-assay; ELISA, enzyme-linked immunosorbent assay; IFU, information for users; LLOQ, lower limit of quantification; LOD, limit of detection; LOQ, limit of quantification.

Immunoassays comparison

In the 82 non-dialysis patients we observed median sclerostin concentrations showing impressive differences between the assays (in pg/mL)—BM: 984 (IQR 648; range 181–4376), TE: 629 (IQR 237; range 274–1961), RD: 154 (IQR 84; range 48–487) and MSD: 36 (IQR 19; range 11–127) (Table 2). Rank correlation (ρ) between assays was all significant, ranging from 0.36 (between BM and MSD) to 0.76 (between RD and TE). Although results seem correlated, the agreement between assays remained quite poor. Indeed, CCC between assays was <0.10 for all comparisons. Only, the comparison between BM and TE reached 0.49, but this result remained low (Figure 1). Bland and Altman analyses confirmed huge differences, a lack of concordance and the absence of systematic differences between the four assays tested (data not shown).

In the 39 dialysis patients, differences in sclerostin concentrations between the various assays were equally impressive. Median concentrations were (in pg/mL) BM: 1976 (IQR 1940; range 362–9717), TE: 1050 (IQR 780; range 389–3502), RD: 169 (IQR 194; range 42–498) and MSD: 23 (IQR 15; range 7–97) (Table 2). Rank correlation (ρ) between assays was significant, ranging from 0.42 (between BM and MSD) to 0.91 (between BM and TE). CCC between assays was very poor for all comparisons (CCC <0.10, except 0.41 between BM and TE). Bland and Altman analyses confirmed huge differences, a lack of concordance and the absence of systematic differences between the four assays tested (data not shown).

Table 2. Clinical and biological characteristics of the cohort

Characteristics	Non-dialysis patients (n =82)	Dialysis patients (n =39)
Age (years)	60 (19)	72 (19)
GFR (mL/min)	71 (44)	NA
Gender (women)	54%	54%
Weight (kg)	76 (25)	NA
Height (cm)	168±11	NA
Parathormone (pg/mL)	26 (26)	NA
Calcium (mmol/L)	2.40±0.11	NA
Phosphore (mmol/L)	0.99 (0.23)	NA
25-OH vitamin D (ng/mL)	23±12	NA
Albumin (g/L)	44 (6)	NA
Sclerostin (BM) (pg/mL)	984 (648)	1976 (1940)
Sclerostin (TE) (pg/mL)	629 (237)	1050 (780)
Sclerostin (RD) (pg/mL)	154 (84)	169 (194)
Sclerostin (MSD) (pg/mL)	36 (19)	23 (15)

Values are represented as median (IQR) or mean ± SD. NA: not available.

Circulating sclerostin versus kidney function

Mean GFR as assessed by iohexol clearance in non-dialysis patients was 72 ± 31 mL/min (range 16–158). A significant negative correlation was found between GFR and sclerostin measured by BM ($r = -0.31$, $P = 0.0045$) and TE ($r = -0.47$, $P < 0.0001$), but no association was found between sclerostin measured by MSD, RD or GFR.

Circulating sclerostin levels were also compared between patients, categorized according to the level of kidney function—Group A: iohexol GFR > 60 mL/min ($n = 50$); Group B: iohexol GFR < 60 mL/min ($n = 32$ CKD) and Group C: dialysis patients ($n = 39$) [Table 3 and Figure 2 (box plot)]. None of the assays showed significant differences in circulating sclerostin between Groups A and B (Table 3). Significantly higher circulating sclerostin concentrations were observed in Group C as compared with Group A or Group B, at least when sclerostin was measured with BM or TE. Sclerostin level measured with

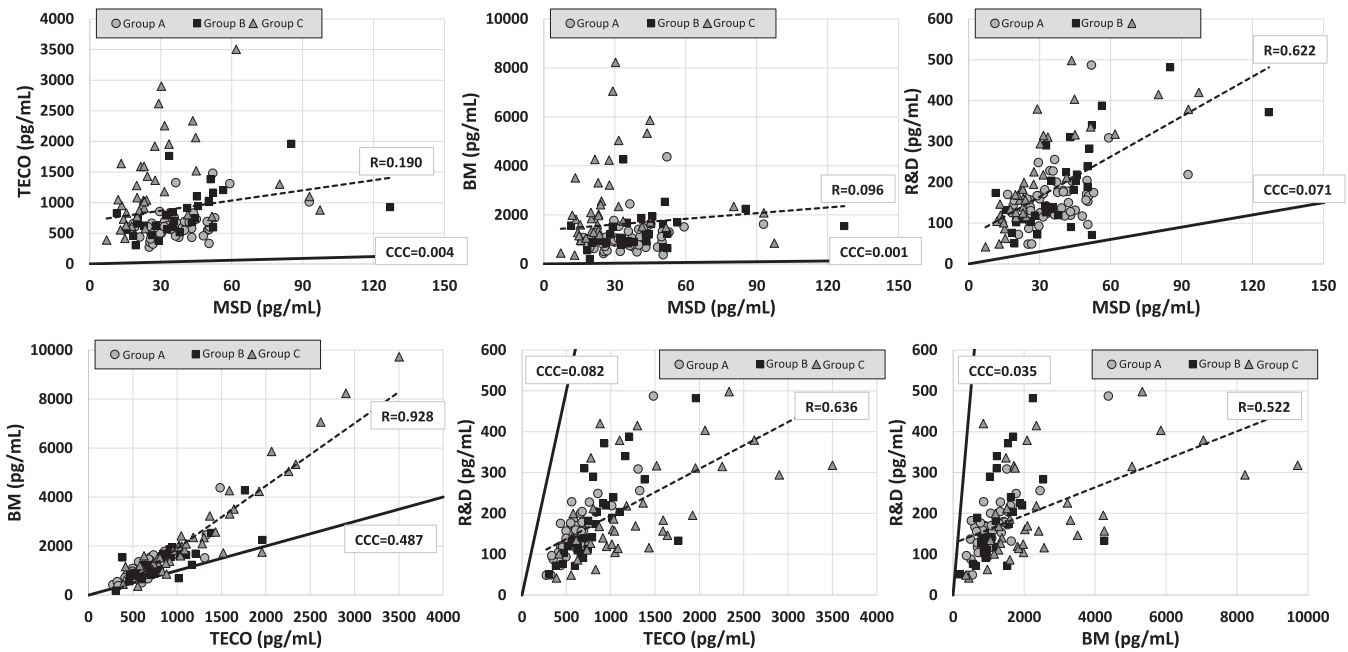

FIGURE 1: CCC and Pearson correlation coefficient (R) between the different assays. The solid straight line is the identity line. The dashed straight line is the linear regression line. The scales of concentrations are very different between assays. Group A: iohexol GFR >60 mL/min (n=50); Group B: iohexol GFR <60 mL/min (n=32); Group C: dialysis patients (n=39).

MSD tended to be lower in Group C as compared with other groups (Table 3, Figure 2).

Circulating sclerostin versus demographic and biological variables

Clinical and biochemical determinants of circulating sclerostin were identified by backward and forward multiple regression in the 82 non-dialysis patients. Importantly, determinants differed according to the immunoassay used to measure circulating sclerostin. With the BM assay, sclerostin was associated with age and weight in the final model. Although sclerostin concentration was significantly correlated with GFR in univariate analysis, this association totally disappeared when age was entered in the model. With the TE assay, PTH and GFR were identified as significant variables above and beyond age and weight. With the RD assay, sclerostin concentration was independently associated with age, gender, PTH and GFR. The determinants explained 23.5, 40.6 and 27.4% of the variability of circulating sclerostin as assessed by the BM, TE and RD assays, respectively. With the MSD assay, no variable was found to be associated with circulating sclerostin.

Impact of a single HD/HDF session on circulating sclerostin levels

A significant decrease of circulating sclerostin levels was observed after a single dialysis session, with the magnitude of the reduction ratio varying between 25 and 46% depending on the assay (Table 4). The reduction ratio differed significantly between MSD and TE, MSD and BM, and MSD and RD. With HDF ($n = 21$), the reduction ratio varied between 25 and 52%, with greater decreases observed with BM and TE. With HD ($n = 18$), the reduction ratio varied between 16 and 38%, with smaller decreases observed with MSD. Compared with HD, HDF was associated with greater sclerostin decreases with TE ($P = 0.033$) and BM ($P = 0.012$), whereas decreases were similar for both modalities with the RD and MSD assays (Table 4).

DISCUSSION

The main findings of the current study are as follows: first, commercially available assays for circulating sclerostin yield markedly discrepant values across the spectrum of kidney dysfunction. Second, immunoassay choice matters when exploring the relationship between circulating sclerostin and

Table 3. Median sclerostin concentrations according to the assay and renal status

	BM	TE	RD	MSD
All ($n = 121$)	1209 (889)	698 (452)	157 (99)	32 (21)
All non-dialysis ($n = 82$)	984 (648)	629 (237)	154 (84)	36 (19)
Iohexol GFR > 60 mL/min (group A) ($n = 50$)	904 (613)	609 (181)	156 (55)	36 (17)
Iohexol GFR < 60 mL/min (group B) ($n = 32$)	1137 (743)	745 (377)	140 (121)	35 (21)
Dialysis (group C) ($n = 39$)	1976 (1940)	1050 (780)	169 (194)	23 (15)

Values are represented as median (IQR) concentrations of sclerostin. All results are expressed in pg/mL.

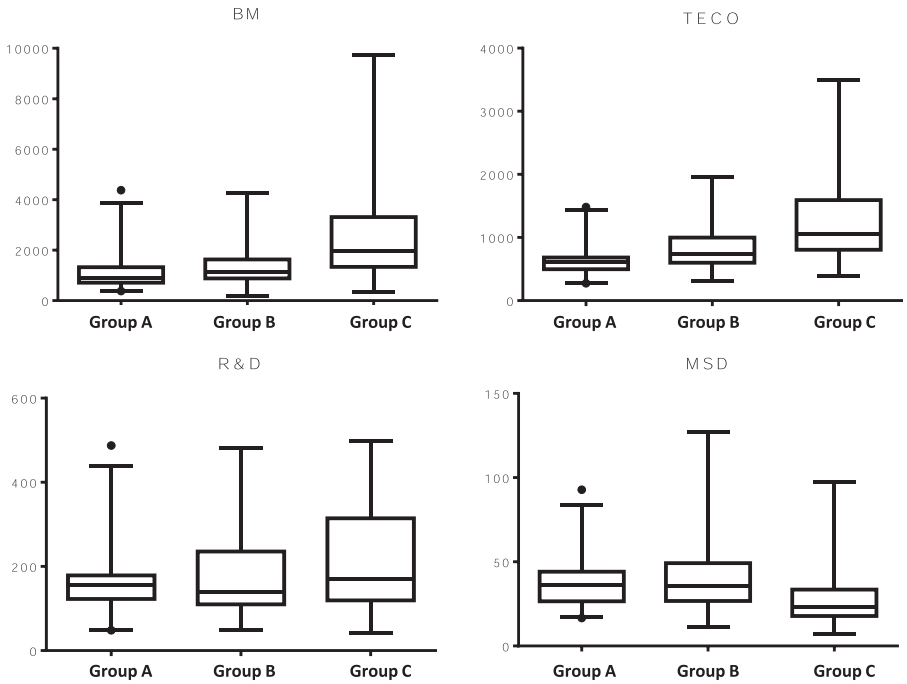

FIGURE 2: Difference in sclerostin levels according to CKD status with the four different assays. Group A: iohexol GFR >60 mL/min (n=50); Group B: iohexol GFR <60 mL/min (n=32); Group C: dialysis patients (n=39).

established or putative determinants, including kidney function, PTH, age and dialytic removal.

The present study confirms and extends previous studies in general and CKD populations identifying major differences between commercially available assays for circulating sclerostin level. For the first time, we performed a head-to-head comparison of four immunoassays and thereby focused on the clinical performance of these assays in individuals with preserved and impaired kidney function [20, 33, 37–41].

Importantly, the between-assay differences were large and not systematic, as demonstrated notably by the CCC analysis, suggesting that specificity rather than calibration issues are implicated. As hypothesized by other authors, differences in assays could be explained by different antibodies used by the manufacturers with no real knowledge of the epitopes these antibodies bind, except maybe for MSD [33, 42]. Cross-reactivity with other proteins cannot be excluded [33]. Alternatively, the antibodies may not only capture the whole intact sclerostin, but also (some of its degradation) fragments (see below). On one hand, the specificity of the MSD assay is presented as better than the others [33, 42]. On the other hand, if the BM, TE and RD enzyme-linked immunosorbent assays (ELISAs) could be considered as 'classical' ones, the instructions provided in the instructions for use in the MSD kit are much more basic and necessitate some adaptation by the laboratory (i.e. the agitation speed is not mentioned, no internal control is provided, some points have to be added in the calibration curve in the physiological measuring range, etc.).

A key question is whether these interassay differences are clinically relevant, that is, whether they impact on the association of the biomarker with established and putative determinants. Extant literature describes the GFR's influence upon sclerostin levels [15–17]. Pelletier *et al.* [16] studied the association between measured GFR and sclerostin in 90 CKD patients and healthy subjects. They found a strong inverse association in simple regression between sclerostin measured with BM and GFR ($r = -0.58$, $P < 0.0001$). Our observations raise questions about the validity of this association: first, the inverse association between GFR and sclerostin measured with BM disappeared in the age-adjusted model. Second, no association was observed between GFR and sclerostin measured with MSD or RD. The inverse association between circulating sclerostin and GFR could be an analytical artifact.

A parallelism can be drawn with other biomarkers such as PTH and procollagen type 1 N propeptide (PoneNP). In human serum, P1NP is present in two major forms, an intact trimeric form and a monomeric one. Some assays recognize both forms ('total P1NP', Roche Elecsys) while other assays recognize the trimeric form only ('intact P1NP', Orion Diagnostica and IDS iSYS). Since only the monomeric form is elevated in CKD patients, the first assay will give higher results in patients with a low GFR, whereas concentrations obtained with the intact assay will not be influenced by GFR [43]. One may thus speculate that TE and BM assays detect both the intact sclerostin molecule and its fragments, while MSD only detects intact sclerostin [20].

Several lines of evidence support this line of reasoning. First, it is evident that the MSD kit systematically gave the lowest sclerostin values (by analogy, biointact/whole PTH assays give lower values than the so-called intact PTH assays, or intact P1NP assay gives lower values than less specific assays also measuring monomeric fragments). Second, Van Lierop *et al.* [44] demonstrated that while synthetic sclerostin fragments (consisting of the loop 1–loop 2–loop 3 without the N- and C-termini, loop 1–loop 3 without the N- and C-termini and loop 2 alone) are not recognized by the MSD assay, they are recognized by the BM assay, likely due to the latter assay's configuration, which employs a polyclonal capture antibody prior to detection with a monoclonal antibody conjugate (BM product insert). The specificity of the MSD antibodies is slightly more restrictive: two capture epitopes (the first to the N-terminal 50 amino acids and a second to the distal C-terminal 42 amino acids) along with three cited epitopes for the tracer (N-, C-terminus and loop 3) [44]. Since it is not yet possible to clearly define by a chromatographic method whether the fragments are detected or not by the different antibodies, one can speculate that these fragments can, to different extents, cross-react or not with these antibodies. Moreover, Van Lierop *et al.* also measured sclerostin concentrations in seven patients with sclerosteosis (bearing mutation in sclerostin gene, SOST) with MSD and BM assays. As expected, they found the MSD assay did not detect any sclerostin in these patients whereas the BM assays detected some sclerostin in three patients, suggesting false positive results [20, 42]. Finally, this hypothesis could also explain, at least in part, one provocative result, i.e. the lower sclerostin concentrations observed in dialysis patients compared with non-dialysis subjects with the MSD assay. The much higher sclerostin values observed in dialysis patients with BM or TE assays [16, 22, 25] could be due to the accumulation of fragments.

Discrepancies are not limited to the relationship between sclerostin and GFR. In CKD populations, circulating sclerostin levels have been associated with various clinical or biological variables [15, 22, 26, 29–31, 45, 46]. Among these, age, GFR and PTH have most often been identified as independent determinants of sclerostin concentration [15, 16, 21, 26, 29–31, 46]. However, the strength and even the direction of the associations often show marked differences. We provide, for the first time,

Table 4. Percentage of median relative decrease (range) in circulating sclerostin concentrations after a single dialysis session

	BM	TE	RD	MSD
Total ($n = 39$)	−46 (−84, +78)	−36 (−54, +15)	−32 (−67, +14)	−25 (−69, +103)
HDF ($n = 21$)	−52 (−65, +37)	−42 (−54, −20)	−31 (−62, +14)	−25 (−53, −6)
HD ($n = 18$)	−38 (−84, +78)	−33 (−51, +15)	−33 (−67, +11)	−26 (−69, +103)
P values (HDF versus HD)	0.012	0.033	0.439	0.833

P-value for Wilcoxon rank sum test.

convincing evidence that specificities of the assay may underlie at least part of the reported discrepancy. Experimental data indicate that PTH may suppress sclerostin expression in osteocytes [47, 48]. The absence of correlation between sclerostin concentrations and PTH observed in the present clinical study with BM and MSD assays questions the precision, if not the reliability, of these assays. In the same way, the absence of any association between sclerostin measured by MSD and any other clinical or biological variables is surprising and questions the reliability of this bioassay. These clinical results are somewhat difficult to reconcile with analytical studies suggesting a better specificity for the MSD assay, which could measure the 'intact' sclerostin [33, 42]. Further studies with this bioassay are clearly needed.

Regarding the sclerostin level response to dialysis, available literature is sparse. Two studies, one with BM [46] and the other with TE [49], found a decrease in sclerostin after one dialysis session. The first study, however, found no difference between HDF and HD techniques but both pre- and post-HDF techniques were included with no minimal volume of ultrafiltration required [46]. Also of interest, a recent study measured sclerostin with the BM assay in 396 patients treated by HD or HDF in a *post hoc* analysis of a randomized study. Pre-dialysis sclerostin concentrations, measured with BM, decreased over time in the HDF group compared with the HD group, suggesting that sclerostin could be more dialyzed in HDF [50]. The present study affords global confirmation that HDF induces a more important decrease in sclerostin levels than one classical dialysis session as assessed by TE or BM assay sclerostin measurement. However, if sclerostin is measured with MSD or RD, no difference in sclerostin concentration between one HDF or HD session was observed. Going back to the hypothesis of an intact dosage of sclerostin, it could be hypothesized that HDF allows the decrease of larger (and potentially inactive) fragments of sclerostin, these fragments being measured by BM or TE but not by MSD or RD. Again, the question of the 'dialyzibility' of sclerostin seems to be influenced by the way the protein is measured.

Our study had several limitations. First, sample size was relatively limited, being constrained by the study's underlying strength, as admitted samples had GFR measured by the rigorous iohexol reference method. Second, despite being kept under the best −80°C storage conditions and assayed at the same time, the samples from the GFR measured and HD patient cohorts were obtained at different times. Pre-analytical sample handling might have introduced some bias, even if the sclerostin stability at −80°C is actually not known [33]. Third, since between-assay differences were the study's primary focus, the magnitude of possible plasma versus serum variances was not addressed [33, 37–40, 46]. Lastly, in the 'before/after' cohort, the *Kt*/*V* results were not available. Also, some clinical and biological outcomes of potential interest (such as FGF-23) were not tested, notably in dialysis patients.

In conclusion, clinical inference with regard to circulating sclerostin is largely influenced by the assay used to measure this biomarker. Which analytical assay is utilized impacts the association between observed sclerostin, PTH, age, GFR and dialysis modality. Although it is reasonable to raise the question of which assay is to be preferred, it should be admitted that we currently do not know. On one side, concentrations obtained with the RD and MSD assays are lower than those obtained with either BM or TE. On the other side, RD also captures, like BM and TE, physiological determinants of circulating sclerostin such as PTH. Lastly, RD is a valid ELISA whereas MSD is analytically less robust. However, none of the assays used in the current study can be considered as a reference method. The results must thus be interpreted carefully until a reference method, such as a sclerostin measurement with mass spectrometry, is available. Until such time, or until true immuno specificity is established, diagnostic results from the assays characterized here—BM, TE, RD and MSD—should be interpreted with caution.

CONFLICT OF INTEREST STATEMENT

F.B. is an employee of DiaSorin, a provider of *in vitro* diagnostic products. Diasorin was not involved in the current study. The results presented in this article have not been published previously in whole or part, except in abstract format.

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