Maria Fusaro*, Maurizio Gallieni, Marianna Noale, Giovanni Tripepi, Davide Miozzo, Mario Plebani, Martina Zaninotto, Giuseppe Guglielmi, Diego Miotto, Fabrizio Fabris, Antonio Piccoli, Maria Teresa Vilei, Stefania Sella, Paolo Morachiello, Fabrizio Stoppa, Maurizio Rossini and Sandro Giannini

The relationship between the Spine Deformity Index, biochemical parameters of bone metabolism and vascular calcifications: results from the Epidemiological VERtebral FRACtures iTalian Study (EVERFRACT) in dialysis patients

Abstract

Background: The Spine Deformity Index (SDI) is a measure of vertebral fractures (VFs), providing information on both their number and severity.

Methods: We evaluated the relationships between SDI and clinical, biochemical and arterial calcification parameters in 387 hemodialysis (HD) patients. VFs, assessed by quantitative vertebral morphometry, and vascular calcifications were identified in the same lateral spinal X-ray. To improve the detection of fracture severity, we created a corrected SDI (c-SDI), by dividing SDI for the number of VFs. We assessed routine biochemistry, bone-Gla-protein (BGP), undercaboxylated BGP (ucBGP), and matrix-Gla-protein (MGP).

Results: VFs prevalence was 55.3%. HD patients with a SDI >1 were more frequently males ($p<0.05$), and had lower BGP ($p<0.01$). Patients with a c-SDI >1 had higher LDL-cholesterol ($p<0.05$) and lower ucBGP ($p<0.05$) and MGP ($p<0.05$). Calcifications of the abdominal aorta (AAoC) were more frequent in patients with SDI >1 ($p<0.05$) and with c-SDI >1 ($p<0.05$). Multivariate logistic regression showed that male sex (OR 1.86, CI 1.20–2.91), age (OR 1.03, CI 1.01–1.05) and albumin ≥3.5 g/dL (OR 0.54, CI 0.31–0.93) were predictors of a SDI >1. Age (OR 1.05, CI 1.03–1.07), LDL-cholesterol (OR 1.74, CI 1.04–2.92) and ucBGP (OR 0.35, CI 0.18–0.70) were associated with c-SDI >1.

Conclusions: We conclude that the severity of VFs was associated with age, atherogenic factors and bone metabolism markers.

Keywords: arterial calcifications; hemodialysis; vertebral fractures.

DOI 10.1515/cclm-2014-0194
Received February 21, 2014; accepted April 19, 2014; previously published online June 4, 2014

***Corresponding author: Maria Fusaro,** MD, PhD, CNR, Neuroscience Institute, Via Giustiniani, 2, 35128 Padua, Italy,
Phone: +39 0498212173, Fax: +39 0498212151,
E-mail: dante.lucia@libero.it
Maria Fusaro and Marianna Noale: National Research Council (CNR), Neuroscience Institute, Padua, Italy
Maurizio Gallieni: Nephrology and Dialysis Unit, Ospedale San Carlo Borromeo, Milan, Italy
Giovanni Tripepi: Clinical Epidemiology and Physiopathology of Renal Diseases and Hypertension, CNR, Institute of Biomedicine, Reggio Calabria, Calabria, Italy
Davide Miozzo and Antonio Piccoli: Nephrology Unit, Department of Medicine, University of Padua, Padua, Italy
Mario Plebani and Martina Zaninotto: Laboratory Medicine Unit, Department of Medicine, University of Padua, Padua, Italy
Giuseppe Guglielmi: Department of Radiology, University of Foggia, San Giovanni Rotondo, Foggia, Italy; and Department of Radiology, Scientific Institute Hospital 'Casa Sollievo della Sofferenza', San Giovanni Rotondo, Foggia, Italy
Diego Miotto: Department of Clinical and Diagnostic Sciences and Special Therapies, Radiology Unit, University of Padua, Padua, Italy
Fabrizio Fabris, Maria Teresa Vilei, Stefania Sella and Sandro Giannini: Clinica Medica 1, Department of Medicine, University of Padua, Padua, Italy
Paolo Morachiello: Nephrology and Dialysis Unit, Hospital of Mestre, Venezia, Italy
Fabrizio Stoppa: Nephrology and Dialysis Unit, Hospital of Adria, Rovigo, Italy
Maurizio Rossini: Rheumatology Unit, Department of Medicine, University of Verona, Verona, Italy

Introduction

Vertebral fractures (VFs) are common in the general population. World Health Organization (WHO) estimated that 1.4 million VFs occurred worldwide in the

year 2000 alone [1]. Epidemiological studies carried out in various countries throughout the world have consistently shown that VFs prevalence steadily increases over time [2–4], reaching high rates in the general population ≥50 years of age (20.3%–29.6%) [3–8]. Since many VFs may be relatively asymptomatic and most subjects may not know that they have experienced them [8, 9], missed diagnoses are common, ranging from 29.5% to 46.5% [7, 9, 10]. Under-reporting of VFs in clinical records is also common [11]. The identification of VFs is important in order to undertake actions aimed to prevent the so-called 'VFs cascade', whereby the occurrence of the first VF is a strong risk factor for subsequent vertebral and non-vertebral fractures [12]. The diagnosis of VF is also important because of its well-recognized association with vascular calcifications [13] and increased risk of mortality [14–18].

Likewise, severity of VFs is relevant from a prognostic point of view. Indeed, severity has been found to be a strong and independent predictor for new vertebral and non-vertebral fractures [19]. Tools that integrate both the number and severity of VFs, thus producing a fracture score, may improve the prognostic assessment of patients. This may be especially true in population subgroups, such as for patients with chronic kidney disease (CKD), in whom bone disorders are very common and the risk of VFs is high [20].

The Spine Deformity Index (SDI) is a summary measure of the VF status, incorporating both the number and severity of VFs in a score. For each vertebra, a visual semiquantitative grade of 0, 1, 2, or 3 is assigned for no fracture, mild, moderate, or severe fracture, respectively. The SDI is then calculated by summing the fracture grades of all vertebrae (T5–L4) [21]. For this reason, and as mentioned before, SDI may be a useful tool for physicians in better characterizing patients with bone fragility [21–27]. However, the evaluation of SDI cannot distinguish between a score generated by multiple mild fractures or fewer severe fractures. For example, a SDI value of 9 might be generated by 9 mild fractures (score 1), where 9×1=9 or by three severe fractures (score 3), where 3×3=9. Thus, we wanted to develop a new score, i.e., corrected-SDI (c-SDI), by dividing the SDI score by the number of fractures, in order to obtain a more precise index of fracture severity (see 'Materials and methods' section).

To our knowledge, the relationships between bone turnover, vascular calcifications and VFs have been poorly addressed in CKD patients. We have already demonstrated that VFs are associated with several biochemical parameters of skeletal metabolism as well as with atherosclerotic changes in patients on maintenance hemodialysis (HD) [28]. The aim of this study was now to evaluate the relationships between the classic SDI and a c-SDI (developed at our center), laboratory parameters of bone metabolism and vascular calcifications within the context of the EVERFRACT study [28].

Materials and methods

Patients

We utilized data collected for the EVERFRACT study, a cross-sectional observational investigation of subjects who underwent HD at 18 Italian Dialysis Centers from 2008 to 2009, designed to determine the prevalence of VFs and vascular calcification. The patient population and the methods of the EVERFRACT study have been already described [28]. Briefly, the EVERFRACT study enrolled 387 adult patients, 244 males and 143 females, aged 64.2±14.1 years, who had been on HD for more than 1 year. We excluded patients who had a life expectancy of <6 months, who had any evidence of cancer (with the exception of basalioma) or who had any condition that, according to the investigators, could interfere with the outcome of the study (e.g., VFs due to acute and accidental traumatism). Patients previously treated with anti-osteoporotic drugs or glucocorticoids were excluded. Patients were not excluded if they were on standard therapy for the management of CKD (including statins, vitamin D analogs, calcimimetics and phosphate binders).

Local Ethics Committee's consent was obtained in compliance with the Italian legislation for observational studies. All patients gave the consent to the use of their medical records for the study in compliance with the Declaration of Helsinki.

Methods

Vertebral fractures and vascular calcifications evaluation

A standardized radiograph of the thoracic and lumbar regions of the spine (T5 to L4) in the latero-lateral view with the patient in the lateral recumbent position was obtained and the radiographs were sent to National Research Council (CNR) of Padua for blinded evaluation in duplicate by two physicians. Any differences were resolved by consensus. VFs were identified by Quantitative Vertebral Morphometry (QVM) using specific software (MorphoXpress, developed by Image Metrics for Procter & Gamble Pharmaceuticals, Rusham Park, Egham, UK). At present no longer available and replaced by Spine Analyzer (Optasia Medical, Cheadle Hulme, Cheadle, Cheshire, UK). We defined a VF as a deformity of the vertebral body due to reduction in one of its dimensions by more than 20%. Fractures were classified as mild (20%–25%), moderate (26%–40%) or severe (>40%) based on the amount of height loss, and scores 1, 2 or 3 were assigned, respectively based on the class of height loss. We calculated the SDI by summing the semiquantitative visual assessment of each VF (score 1, 2 or 3); SDI ranges

were from 0 to 36 (minimum and maximum value: 0–3, ×12 vertebrae) [21–27]. However, the evaluation of SDI cannot distinguish between a score generated by multiple mild fractures or fewer severe fractures. For example, a SDI value of 9 might be generated by 9 mild fractures (score 1), where 9×1=9 or by three severe fractures (score 3), where 3×3=9. To correct this possible bias, we divided the SDI score by the number of fractures, in order to obtain a more precise index of fracture severity (c-SDI). In the previous example, the c-SDI will be 1 (9:9=1) in the first case and 3 (9:3=3) in the second one. In other words, the higher is the c-SDI, the worst the severity of fractures.

We also used the thoraco-lumbar radiograph to calculate the vascular calcifications score quantifying the length of the calcium deposits along the abdominal aortic wall (AAoC, mild 0.1–5 cm, moderate 5.1–10 cm, and severe >10 cm) [29]. We also determined whether VCs were visible in the iliac arteries calcifications (IAC), creating a score as follows: mild 0.1–3 cm, moderate 3.1–5 cm, and severe >5 cm, as previously published [28].

Laboratory investigations

Fasting blood samples were obtained from all patients. Serum calcium, phosphorus, alkaline phosphatase (ALP), total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides, albumin, C-reactive protein (CRP), creatinine and blood nitrogen were assayed by standard methods. We then calculated KT/V (K=Dialyzer Clearance, T=dialysis time; V=Volume of distribution of urea) measured as single pool. Assays were performed on fresh plasma at the local study Centre of Laboratory Medicine.

We also measured: parathyroid hormone (PTH), 25(OH)D, total bone-Gla-protein (BGP), undercarboxylated BGP (ucBGP) and total matrix-Gla-protein (MGP) at the central laboratory of Padua (fresh or frozen plasma, see Appendix).

Statistics

Normally distributed data were expressed as mean±SD, not normally distributed data as median and interquartile (IQ) range and binary variables were summarized as absolute frequency and percentage. Two groups of subjects were defined, considering the median for SDI: subjects with a SDI>1, subjects with a SDI≤1. Two further groups were defined considering c-SDI>1 versus c-SDI≤1. The association of general characteristics, biochemical parameters and group of subjects were evaluated using the χ^2-test for categorical variables, the generalized linear model (Welch's ANOVA in case of heteroschedasticity) for quantitative variables normally distributed and the Mann-Whitney's test for not-normally distributed variables. Normality of the distribution was tested through the Shapiro-Wilk test. The associations between AAoC, IAC and SDI, and between AAoC, IAC and c-SDI, were analyzed by univariate and by multivariate logistic regression models. For multivariate models, any significant variable with a p≤0.20 in the univariate model (among sex, age, smoking status, alcohol consumption, BMI, biochemical parameters, diseases) were introduced into a multivariate model using the stepwise selection method. A p-value<0.05 was considered statistically significant. We analyzed data using SAS statistical package, release 9.2 (SAS, Cary, NC, USA) and SPSS for Windows, Version 9.0.1. (Chicago, IL, USA).

Results

In our 387 patients, VFs prevalence was 55.3% (n=214). Patients with VFs were significantly older than those without (66.7±13.1 vs. 61.0±14.7 years, p<0.001) and more frequently males (59.8% vs. 40.2%, p<0.02). Most of the VFs were mild (47%) or moderate (23.5%). They were in large majority mild (35.8%) or moderate (17.4%) wedge dorsal fractures. The mean SDI was 1.4±1.74 (median 1; IQ range 0–2). The mean c-SDI was 0.74±0.75 (median 1; IQ range 0–1.25, Figure 1). As shown in Table 1, the contribution of severe fractures was generally low through T5–T10, while it was higher through T11–L3. The severity of fractures was pointed out by c-SDI only. Indeed, while the SDI did not differ between T5 and T10 (41.0±19.7) and T11 and L3 (51.7±26.2, p=ns), the c-SDI was significantly higher between T11 and L3 (1.43±0.04) as compared to T5 and T10 (1.26±0.04, p<0.001). The prevalence of AAoC in dialysis patients was 80.6%, whereas for IAC it was 55.6%.

The general characteristics and biochemical parameters in patients with a SDI≤1 as compared to those with a SDI>1 are reported in Table 2. HD patients with a SDI>1 were more frequently males, were older and had lower serum albumin values. BGP serum levels were lower in these subjects as compared to those with a SDI≤1. In addition, calcifications of the abdominal aorta, but not those of IACs were more frequent in patients with SDI>1. When comparing patients based on the c-SDI (Table 3), we observed that those with a c-SDI>1 were older, and had higher levels of total cholesterol as well as LDL-cholesterol. BGP levels tended to be lower in these patients, even if not significantly. However, median ucBGP and MGP were significantly lower in patients with a c-SDI>1. Calcifications of the abdominal aorta, but not those of IACs were more frequent in patients with c-SDI>1.

In the univariate logistic regression model, SDI (>1 vs. ≤1) was significantly associated with AAoC, but not with IAC. However, the multivariate model (Table 4) showed that only male sex, age and albumin ≥3.5 g/dL were significant predictors of a SDI >1. In the univariate logistic regression, c-SDI score (>1 vs. ≤1) was significantly associated with AAoC and with IAC. These results were not confirmed by the multivariate model (Table 5), in which a significant association with c-SDI >1 was found for age, LDL-cholesterol and ucBGP.

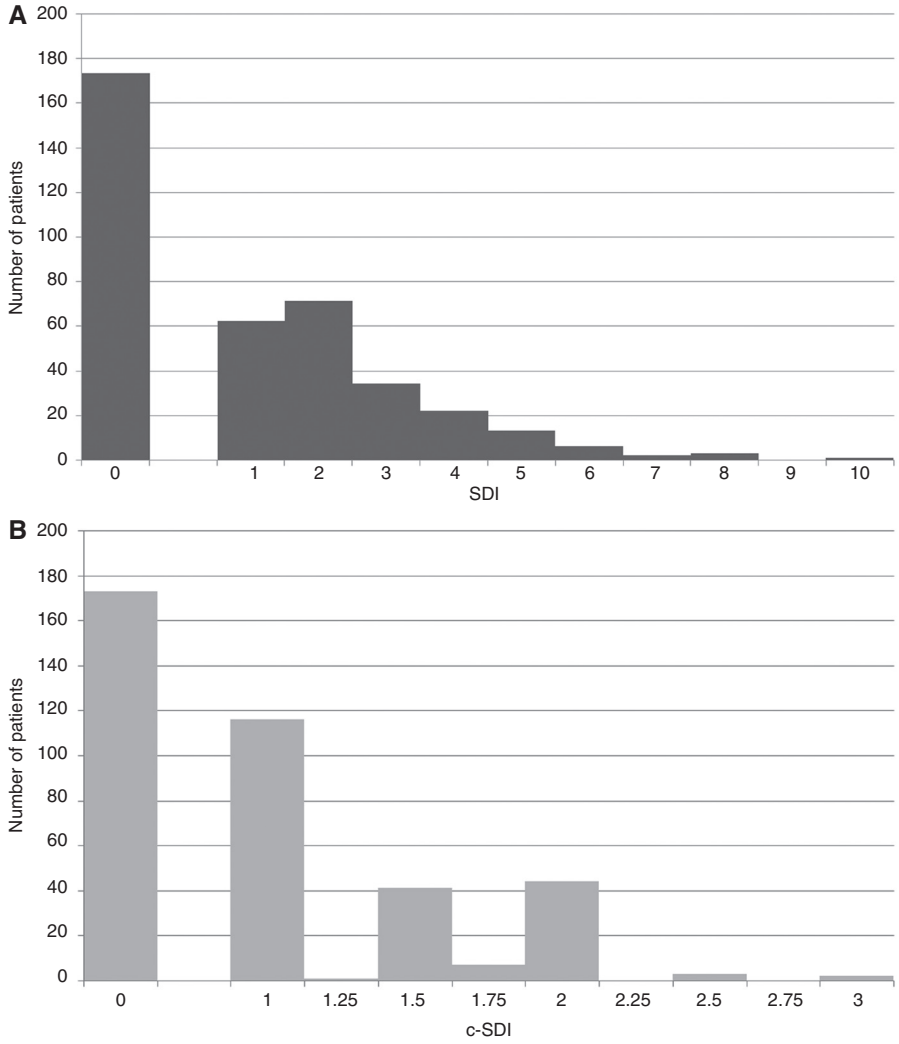

Figure 1 Distribution of Spine Deformity Index (SDI, panel A) and corrected-Spine Deformity Index (c-SDI, panel B) in patients on maintanance hemodialysis are reported.

Table 1 Mild, moderate, and severe vertebral fractures, as well as SDI and c-SDI by vertebra for all patients are reported. c-SDI provides a more precise index as compared to SDI, which is not able to fully distinguish between a score generated by multiple mild fractures or by fewer severe fractures.

Vertebra	Mild VFs score 1, n (%)	Moderate VFs score 2, n (%)	Severe VFs score 3, n (%)	Spine Deformity Index, SDI	Corrected Spine Deformity Index, c-SDI
T5	24 (6.0%)	7 (1.7%)	0 (0.0%)	38	1.23
T6	33 (8.3%)	14 (3.5%)	1 (0.2%)	64	1.33
T7	38 (9.6%)	12 (3.0%)	0 (0.0%)	62	1.24
T8	35 (8.8%)	10 (2.5%)	0 (0.0%)	55	1.22
T9	26 (6.5%)	8 (2.0%)	1 (0.2%)	45	1.29
T10	19 (4.7%)	8 (2.0%)	0 (0.0%)	35	1.30
T11	30 (7.5%)	14 (3.5%)	3 (0.7%)	67	1.43
T12	35(8.8%)	17 (4.2%)	2 (0.5%)	75	1.39
L1	19 (4.7%)	12 (3.0%)	2 (0.5%)	49	1.48
L2	4 (1.0%)	3 (0.7%)	2 (0.5%)	16	1.78
L3	2 (0.5%)	3 (0.7%)	0 (0.0%)	8	1.60
L4	13 (3.2%)	4 (1.0%)	0 (0.0%)	21	1.24
Total	278	112	11	Σ 535	Average 1.38

Table 2 General characteristics and biochemical parameters in patients with a SDI≤1 as compared to those with a SDI>1.

	Patients with SDI≤1 (n=235)	Patients with SDI>1 (n=152)	p-Value
Sex, male, n (%)	136 (57.9)	106 (69.7)	0.0185
Age, years, mean±SD	62.1±14.5	67.3±12.8	<0.0001
Body mass index, kg/m^2, mean±SD	24.8±4.3	25.4±4.6	0.8132
Ca, mmol/L, mean±SD	2.28±0.17	2.29±0.25	0.4713
P, mmol/L, mean±SD	1.56±0.42	1.5±0.39	0.4050
ALP, UI/L, median (IQ)	83 (65, 111)	82 (63, 109)	0.5585
PTH, pg/mL, median (IQ)	244 (144, 400)	231 (131, 355)	0.9635
25(OH)D, ng/mL, median (IQ)	29 (20, 45)	28 (18, 44)	0.4017
Total cholesterol, mmol/L, mean±SD	4.30±1.06	4.48±1.06	0.3267
HDL-cholesterol, mmol/L, mean±SD	1.09±0.31	1.09±0.34	0.7211
LDL-cholesterol, mmol/L, mean±SD	2.38±1.01	2.51±0.88	0.7037
Triglycerides, mmol/L, median (IQ)	1.64 (1.28, 2.28)	1.70 (1.21, 2.44)	0.5853
Albumin, g/dL, mean±SD	3.88±0.42	3.73±0.57	0.0225
CRP, mg/L, median (IQ)	1.58 (0.46, 5.25)	1.85 (0.57, 5.00)	0.2580
KT/V, mean±SD	1.26±0.26	1.24±0.28	0.4581
BGP, mcg/L, median (IQ)	203 (106, 355)	147 (82, 270)	0.0091
ucBGP, mcg/L, median (IQ)	12.1 (4.6, 19)	9.8 (4.4, 15.9)	0.1697
MGP, nmol/L, median (IQ)	20.1 (13, 30.9)	17.8 (12.6, 30.5)	0.2394
AAoC, n (%)	60 (25.5)	56 (36.8)	0.0177
IAC, n (%)	28 (11.9)	26 (17.1)	0.1501

AAoC, calcifications of the abdominal aorta; BGP, bone-Gla-protein; IAC, iliac arteries calcifications; MGP, matrix-Gla-protein; ucBGP, undecarboxylated BGP.

Table 3 General characteristics and biochemical parameters: comparison between patients with a c-SDI≤1 and those with a c-SD≥1.

	Patients with c-SDI≤1 (n=289)	Patients with c-SDI >1 (n=98)	p-Value
Sex, male, n (%)	177 (61.3)	65 (66.3)	0.3692
Age, years, mean±SD	62.3±14.4	69.6±11.4	<0.0001
Body mass index, kg/m^2, mean±SD	25.1±4.5	24.9±4.3	0.6683
Ca, mmol/L, mean±SD	2.29±0.17	2.27±0.29	0.3226
P, mmol/L, mean±SD	1.56±0.41	1.48±0.40	0.1012
ALP, UI/L, median (IQ)	83 (64, 110)	84 (65, 115)	0.6288
PTH, pg/mL, median (IQ)	244 (140, 400)	232 (157, 343)	0.8676
25(OH)D, ng/mL, median (IQ)	29 (20, 44)	26 (18, 45)	0.2513
Total cholesterol, mmol/L, mean±SD	4.30±1.06	4.56±1.06	0.0491
HDL-cholesterol, mmol/L,mean±SD	1.09 ±0.34	1.11±0.34	0.6300
LDL-cholesterol, mmol/L, mean±SD	2.38±0.96	2.62±0.93	0.0386
Triglycerides, mmol/L, median (IQ)	1.64 (1.25, 2.29)	1.73 (1.29, 2.42)	0.3467
Albumin, g/dL, mean±SD	3.84±0.47	3.76±0.55	0.1617
CRP, mg/L, median (IQ)	1.70 (0.47, 5.50)	1.50 (0.56, 3.30)	0.5810
KT/V, mean±SD	1.24±0.26	1.27±0.29	0.3727
BGP, mcg/L, median (IQ)	195 (104, 319)	148 (81, 297)	0.0689
ucBGP, mcg/L, median (IQ)	11.8 (4.7, 19.8)	9.54 (4.02, 15.8)	0.0435
MGP, nmol/L, median (IQ)	20.1 (13.16, 31.95)	15.52 (12, 25.5)	0.0315
AAoC, n (%)	78 (27.0)	38 (38.8)	0.0278
IAC, n (%)	36 (12.5)	18 (18.4)	0.1445

AAoC, calcifications of the abdominal aorta; BGP, bone-Gla-protein; IAC, iliac arteries calcifications; MGP, matrix-Gla-protein; ucBGP, undecarboxylated BGP.

Table 4 Logistic regression model with outcome SDI>1 versus SDI≤1. Stepwise selection method among significant variable (p≤0.20) in the univariate model.

	OR	95% CI	p-Value
Sex, male	1.86	1.20–2.91	0.0067
Age, years	1.03	1.01–1.05	0.0003
Albumin ≥3.5 g/dL	0.54	0.31–0.93	0.0259

Table 5 Logistic regression model with outcome c-SDI>1 versus c-SDI≤1. Stepwise selection method among significant variable (p≤0.20) in the univariate model.

	OR	95% CI	p-Value
Age, years	1.05	1.03–1.07	<0.0001
LDL cholesterol ≥2.34 mmol/L	1.74	1.04–2.92	0.0354
ucBGP≥17.2 mcg/L	0.35	0.18–0.70	0.0025

ucBGP, undecarboxylated BGP.

Discussion

Several studies of our and other groups have already demonstrated that VFs are quite common in patients on maintenance HD and associated with vascular calcifications [13, 28]. To our knowledge, this is the first study attempting to evaluate the association between the SDI with several factors involved in bone and vascular disease in patients with end-stage renal failure. SDI is a measure of both VFs number and severity, widely recognized as a good diagnostic and prognostic index [21–23]. In this view, it not surprising that patients with both SDI and c-SDI>1 were older and that age was a significant predictor of both number and severity of VFs. Indeed, age is a well-recognized fracture risk factor.

We also found that SDI>1 was significantly more frequent in male sex. This could be rather surprising considering that VFs are more common in females than in males, at least in the general population [2]. However, we already reported a higher frequency of VFs in hemodialyzed men as compared to women [28]. These results are also consistent with another published report of our group [30] on kidney transplant recipients, demonstrating a higher prevalence of VFs in men. No comprehensive explanations can be provided for these findings. An intriguing possibility is related to the finding of different BGP and ucBGP serum levels in patients with SDI and c-SDI>1 as compared to those with SDI and c-SDI≤1. Osteocalcin, the most abundant non-collagenous bone matrix protein, is a small γ-carboxyglutamate protein preferentially expressed by osteoblasts [31]. Even if its function has been extensively studied and its ability to regulate bone mineralization and turnover appears certain, the exact role of osteocalcin in bone has not yet fully elucidated [31]. However, it is believed that the carboxylation of osteocalcin, with vitamin K as an important cofactor, confers to this protein biologic activity in bone tissue, while the undecarboxylated portion (ucBGP) seems to be less important for bone turnover and mineralization processes [31]. More recently, several extraskeletal functions have been attributed to osteocalcin, such as an involvement in glucose and adipose tissue metabolism as well as in the vascular calcification process [31]. Noteworthy, ucBGP seems to be particularly bio-active outside the skeleton and is also involved in the regulation of fertility, only in males. Indeed, it has been found that ucBGP increases testosterone biosynthesis in Leydig cells [32]. Testosterone is believed a strong determinant of bone health in males because it has anabolic effects on bone, stimulating the proliferation of osteoblast progenitors and the differentiation of mature osteoblasts [32]. We did not measure testosterone levels in our hemodialyzed men. However, it seems possible that the lower values of ucBGP we found in patients with higher proportion and severity of VFs might have exerted their negative effects on bone by decreasing testosterone production by testes, thus increasing bone fragility in these patients. This hypothesis is also in keeping with the well-known reduction in testosterone levels reported by several authors in CKD patients [33]. Further studies are warranted to elucidate this interesting point.

The present study also confirms that higher levels of serum albumin are protective against VFs: serum albumin values ≥3.5 g/dL decreased by almost 50% the probability of SDI >1. These results are in keeping with other studies, carried out in the general population as well as in patients with CKD, reporting that low serum albumin may expose patients to increased fracture risk [34–36]. We found that aortic calcifications were approximately 30% more frequent in patients with both SDI and c-SDI>1. This is not surprising because these indexes are a measure of VFs prevalence and the association between aortic calcifications and VFs has been already described in several reports both in healthy adults and in hemodialyzed patients [37]. In addition, patients with c-SDI>1 showed lower levels of MGP and BGP as well as higher serum LDL-cholesterol. MGP is a well-recognized potent inhibitor of arterial calcifications [38] and LDL-cholesterol is still considered one of the major determinants of atherosclerosis. Furthermore, some studies showed an association between LDL-cholesterol with BMD [39] and VFs [40]. Recently, Confavreux et al. reported that higher BGP serum levels

were associated with lower progression of aortic calcifications and increased survival in elderly men [41]. Taken together, these data strengthen the concept that bone fragility and vascular calcification may share some common pathways and that the presence of one of these two should prompt the clinicians to look for the other.

The SDI is a summary measure of the VF status, incorporating both number and severity of VFs in a unique score. However, once calculated, SDI cannot distinguish between a score generated by multiple mild fractures or fewer severe fractures. On the contrary, c-SDI, which is obtained dividing the SDI by the number of detected fractures, gives more information about the severity of VFs. Indeed, we found that c-SDI values were significantly higher between T11 and L3, where most severe fractures were concentrated, than between T5 and T10, while this was not true for SDI. We are aware that the superiority of c-SDI as compared to SDI should be assessed through the evaluation of their prognostic utility. However, the cross-sectional design of our study does not allow us to address this specific point and further work in this direction is warranted.

We are well aware that this study has several limitations. The cross-sectional design did not allow us to draw any conclusions about the relationships between number and severity of VFs with morbidity and mortality [14–18] in CKD patients. In addition, the absence of DXA measurements of bone density and bone quality parameters, such as trabecular bone score could have affected a more comprehensive evaluation of these patients.

In conclusion, c-SDI was associated with biochemical markers of bone metabolism (ucBGP) and vascular calcifications (LDL-cholesterol). Aortic calcifications were more prevalent in hemodialyzed patients with higher frequency and severity of VFs. Further study should aim at a more comprehensive view of these two important processes.

Acknowledgments: We would like to acknowledge the contribution of Dr. S.A. Jamal, University of Toronto, Women's College Hospital and Women's College Research Institute of Toronto, Ontario, Canada, for her precious work in reviewing the manuscript.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.

Appendix

Parathyroid hormone (PTH)

The method for quantitative determination of PTH in serum was the automated LIAISON® N-Tact® PTH Assay 310910 (DiaSorin Inc., Stillwater, MN, USA), which is a direct, two-site, sandwich type chemiluminescence immunoassay (CLIA) carried out on the LIAISON® (DiaSorin Inc.) instrument. During incubation, the solid phase, coated with specific antibodies against 39-84 region of PTH, binds the molecules in the samples and is subsequently bound by a second antibody for the 1-34 region conjugated to an isoluminol-derivative. The starter reagent is then added, leading to a chemiluminescent signal that is proportional to the concentration of PTH present in the samples. The analytical sensitivity is 1 pg/mL, and the intra- and inter-assay CV were 3.7%–6.3% and 3.5–5.3%, respectively.

25-OH vitamin D

For quantitative determination of total 25-OH vitamin D (both D_2 and D_3 form) in serum, we used the automated LIAISON® 25 OH Vitamin D TOTAL Assay 310600, which is a direct competitive CLIA executed on the LIAISON® (DiaSorin Inc.) instrument. During the first incubation, 25-OH vitamin D is dissociated from its binding protein and binds to the specific antibody on the solid phase. Then the tracer (vitamin D linked to isoluminol derivative) is added. After a second incubation and the wash cycle for unbound material, the starter reagents are added. The flashlight signal is inversely proportional to the concentration of 25-OH vitamin D present in the samples. The analytical sensitivity is <4 ng/mL, and the intra-assay coefficients of variation (CV) have been found to range between 2.9% and 5.5%, while the inter-assay CV is 6.3%–12.9%.

Osteocalcin (BGP)

The method for quantitative determination of total osteocalcin in serum was the automated LIAISON® Osteocalcin Assay 310950 (DiaSorin Inc.), which is a direct, two-site, sandwich type CLIA executed on the LIAISON® (DiaSorin Inc.) instrument. The osteocalcin in the samples binds the mouse antibody, coating the solid phase, and is subsequently bound by isoluminol conjugated antibody. After the incubation, the unbound material is removed by wash cycle, and then the starter reagents are added.

The flashlight signal is proportional to the concentration of osteocalcin present in the samples. The analytical sensitivity is <0.3 mcg/L, and the intra-assay CV is 3%–8%, while the inter-assay CV is 4%–9%.

Undercarboxylated osteocalcin (ucBGP)

For quantitative determination of the undercarboxylated form, we used Glu-osteocalcin EIA Kit MK118 (Takara Bio Inc., Otsu, Shiga, Japan), a manual solid phase EIA based on a sandwich method that utilizes two mouse monoclonal anti-ucBGP antibodies to detect uc-BGP by a two-step procedure. One of the mouse monoclonal anti-undercarboxylated BGP is immobilized onto the microtiter plate and blocked against non-specific binding. Samples are added to each well and incubated. The second step is to wash the plate and to add the second anti-BGP labeled with peroxidase (POD). The reaction between POD and substrate (H_2O_2 and 3,3′, 5,5′ tetramethylbenzidine) results in color development with intensities proportional to the amount of uc-BGP present in the samples. The analytical sensitivity is 0.25 mcg/L, and the intra- and inter-assay CV are 4.4%–6.7% and 5.7%–9.9%, respectively.

Matrix-Gla-protein (MGP)

The quantitative determination of MGP was performed using the Human MGP-Matrix Gla ProteinKit (Biomedica Medizinprodukte GmbH & Co KG, Vienna, Austria). It is a manual competitive ELISA method designed to detect MGP in serum. During the first incubation, the MGP in the samples is bound by the specific antibody coated to the microtiter plate. Moreover, the tracer (biotinylated synthetic MGP) is added to each well. Then, after a wash cycle to remove the unbounded tracer, the conjugate [streptavidin-linked horseradish peroxidase (HRPO)] is added to each well. The reaction between HRPO and substrate TMB (3,3′,5,5′-tetramethylbenzidine) develops color with intensities inversely proportional to the MGP present in samples. The analytical sensitivity is 0.3 nmol/L, and the intra- and inter-assay CV are 5%–6% and 7%–9%, respectively.

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