Clinical Kidney Journal

ckj

OXFORD

Leading European Nephrology

Clinical Kidney Journal, 2017, vol. 10, no. 3, 389–396

doi: 10.1093/ckj/sfw145
Advance Access Publication Date: 15 February 2017
Original Article

ORIGINAL ARTICLE

Is chronic kidney disease-mineral and bone disorder associated with the presence of endothelial progenitor cells with a calcifying phenotype?

Giuseppe Cianciolo[1], Irene Capelli[1], Maria Cappuccilli[1], Anna Scrivo[1], Chiara Donadei[1], Antonio Marchetti[2], Paola Rucci[2] and Gaetano La Manna[1]

[1]Department of Experimental Diagnostic and Specialty Medicine (DIMES), Nephrology, Dialysis and Renal Transplant Unit, St Orsola Hospital, University of Bologna, Bologna, Italy and [2]Department of Biomedical and Neuromotor Sciences, Unit of Hygiene and Biostatistics, University of Bologna, Bologna, Italy

Correspondence and offprint requests to: Gaetano La Manna; E-mail: gaetano.lamanna@unibo.it

Abstract

Background: Chronic kidney disease-mineral and bone disorder (CKD-MBD) has been implicated in vascular calcification pathogenesis. CKD-MBD results in alterations in the number and function of circulating endothelial progenitor cells (EPCs), physiological regulators of angiogenesis and vessel repair, commonly defined as proangiogenic progenitor cells (PACs) by the antigen pattern CD34+CD133+KDR+CD45– and putative EPCs by the pattern CD34+CD133−KDR+CD45–. These cells might acquire a calcifying phenotype in CKD-MBD, expressing mineralization biomarkers. We investigated the expression of vitamin D receptor (VDR) and osteocalcin (OC) on EPCs of healthy individuals and haemodialysis patients, and their possible associations with circulating biomarkers of inflammation and vascular calcification.

Methods: We compared EPC counts, expressing VDR or OC, in 23 healthy subjects versus 53 haemodialysis patients, 17 of them without vitamin D receptor agonist (VDRA) therapy and 35 treated with calcitriol ($n = 17$) or paricalcitol ($n = 18$). The correlations with serum levels of inflammatory and calcification indexes were also analysed.

Results: All subsets expressing VDR or OC were significantly higher in haemodialysis patients compared with healthy controls, but PACs were increased only in VDRA treatment subgroup, while putative EPCs showed a similar rise also in untreated patients. In VDRA-untreated patients, OC+ PACs correlated positively with calcium levels, while in VDRA-treated patients, VDR+ PACs correlated positively with interleukin 6 levels, and OC+ PACs correlated positively 25-hydroxyvitamin D levels.

Conclusions: Our data suggest that in CKD-MBD, EPCs undergo an endothelial-to-procalcific shift, representing a risk factor for vascular calcification. A link between mineral disorders and vitamin D replacement therapy emerged, with potential adverse effects for CKD patients.

Key words: haemodialysis, osteocalcin, vascular calcification, vitamin D receptor, vitamin D receptor agonist therapy

Received: October 11, 2016. **Accepted:** December 2, 2016

Introduction

There is a tight physiopathological link between bone turnover, vascular calcification and cardiovascular events, in the general population and in patients with chronic kidney disease (CKD) [1]. CKD accelerates medial and intimal calcification development, which both rapidly further worsen when the patients undergo haemodialysis treatment [2]. Vascular calcification is now recognized as an active process retracing the normal sequence of osteoblast/osteoclast signalling and activities on bone surfaces. Its pathogenesis is a multifactorial process, feasibly determined by the contribution of inflammation, oxidative stress and above all alterations of bone and mineral metabolism, collectively known as CKD-mineral and bone disorder (CKD-MBD) [3]. The recent identification of circulating calcifying cells raises the question of whether these cells should be added to the list of vascular calcification promoters and what if any relationship exists with MBD. The pool of circulating calcifying cells includes several, likely interrelated, osteogenic cell phenotypes of mesenchymal and haematopoietic origin: mesenchymal osteoprogenitor cells, myeloid calcifying cells, circulating calcifying CD34 progenitor cells and endothelial progenitor cells (EPCs) [4]. EPCs are a subgroup of bone marrow-derived blood mononuclear cells able to circulate, proliferate and differentiate into mature endothelial cells, essential for angiogenesis and vessel repair. The major source of EPCs is the bone marrow: although several putative EPCs phenotypes differ in lineage origin and function, EPCs are thought to descend from haematopoietic stem/progenitor cells (HSCs). Thus, EPCs originating from this lineage are characterized by both stemness markers, CD34 and CD133, and endothelial markers, namely vascular endothelial growth factor receptor-2 (KDR). The co-expression of the stem cell antigen, CD133, increases specificity for EPCs. This antigen is a marker of stemness and it is not expressed by mature endothelial cells, while the lack of CD45, generally considered a specific pan-leukocyte marker, identifies phenotypes restricted to the endothelial lineage. The characterization of EPCs based on the identification of cell surface antigens requires simple, rapid and reproducible methods: flow cytometry is the gold standard for this purpose [5].

The term EPC comprises a heterogeneous group of cells including a subset of cells known as proangiogenic progenitor cells (PACs) and identified by various combinations of CD34, CD133 and KDR [6, 7]. Differentiation begins when the cells are mobilized from the bone marrow into the bloodstream. During this process, immature cells—characterized by the CD34+CD133+KDR+phenotype—upon entering circulation, start to express more endothelial cell markers and lose the CD133 [7].

Currently, the CD34+CD133+KDR+CD45– and CD34+KDR+CD45– subsets appear to be the best compromise in terms of sensitivity, specificity and reliability to quantify EPCs in the clinical setting [7]. The first subset (CD34+CD133+KDR+CD45–) defines PACs, which promote angiogenesis via a paracrine mechanisms: these cells feasibly acquire proangiogenic ability after recruitment into sites of neovascularization and stimulation by an angiogenic milieu. The second subset (CD34+KDR+CD45–) has angiogenic properties and characterizes putative EPCs [6]. PACs and putative EPCs are the terms used in this paper to identify these cells subsets (Table 1).

Recent data have demonstrated that circulating CD34 progenitor cells, PACs and putative EPCs can also express bone-related proteins, namely osteocalcin (OC) and/or bone alkaline phosphatase (BAP). OC is a noncollagenous bone protein implicated in bone mineralization and calcium homeostasis, while BAP is a glycoprotein found on osteoblast surface, essential for mineralization process [8].

Circulating EPCs expressing OC, implicated in coronary artery disease and calcific aortic stenosis, are retained in the coronary circulation of patients with coronary disease and diabetes [9, 10]. Although many factors have been identified as potential triggers of endothelial dysfunction in CKD patients, little is known about the possible changes in EPCs, the presence of circulating EPCs expressing OC and the involvement of CKD-MBD. This is a central issue, since vitamin D receptor (VDR), parathyroid hormone receptor (PTHR) and calcium-sensing receptor (CaSR) have been found on endothelial cells, EPCs and HSCs. Some studies have also proven a protective role of vitamin D and VDR agonists (VDRA) on EPCs' number and function in diabetic and CKD patients [11–13].

In view of the above, this study was undertaken to: (i) compare the relative count of PACs and putative EPCs expressing VDR and OC between healthy individuals and haemodialysis patients; and (ii) evaluate the association of laboratory parameters and biochemical markers involved in inflammatory, MBD and VDRA therapy with PACs and putative EPCs expressing a percentage of VDR and OC.

Materials and methods

Ethics statement

The study recruited 53 dialysis patients and 23 healthy volunteers. The protocol was approved by the local ethics committee (S. Orsola-Malpighi University Hospital, Bologna, Italy, protocol number: 053/2010/O/Oss) and written informed consent was obtained from all subjects in accordance with the Helsinki Declaration.

Patients

From January to December 2013, 53 Caucasian patients who had been on haemodialysis at our centre for at least 6 months were recruited, with a control group of 23 healthy volunteers matched for sex and age. The patients underwent regular bicarbonate haemodialysis treatment three times a week with polysulfone membrane; Kt/V (according to Daurgirdas' second logarithmic formula) was 1.3 ± 0.3; the diuresis was <200 mL/day. Treatment schemes were established on the basis of bone mineral disorder indexes: patients did not receive any VDRA therapy (if PTH was <200 pg/mL), 17 patients were treated with oral calcitriol (if PTH was >200 and <600 pg/mL) and 18 patients intravenous paricalcitol (if PTH >600 pg/mL). Each patient

Table 1. Cell phenotypes: surface antigen pattern, nomenclature and function

Surface markers	Cell subpopulation	Function
CD34+	Haematopoietic stem progenitor cells	Maintenance and replenishment of all blood cell types in the bone marrow
CD34+CD133+KDR+CD45−	Proangiogenic progenitor cells	Paracrine angiogenic effects
CD34+CD133−KDR+CD45−	Putative endothelial progenitor cells	Angiogenic properties

followed the same therapy over time in the 6-month period prior to enrollment.

Inclusion criteria were: (i) age > 18 years; (ii) clinical stability for at least 3 months before study entry; and (iii) functioning arteriovenous fistula as vascular access. Exclusion criteria were: (i) active infections; (ii) cardiovascular events in the previous 6 months; (iii) malignancy; (iv) active gastrointestinal bleeding; (v) bone fractures in the last 12 months; (vi) Paget's disease; (vii) bisphosphonate therapy; (viii) familial dyslipidaemia; (ix) uncontrolled hypertension (target blood pressure persistently >140/90 mmHg); and (x) ACE (angiotensin-converting enzyme) inhibitors or angiotensin II receptor antagonists, or calcium mimetic therapy.

The following demographic, clinical and biochemical parameters were collected: sex, age, dialysis vintage, CKD duration, diabetes, coronary computed tomography angiogram (CTA), erythropoietin treatment regimen, haemoglobin, reticulocytes, serum creatinine, lipid profile, calcium, phosphate, albumin, total alkaline phosphatase (ALP), iron, ferritin, PTH, C-reactive protein (CRP), BAP and 25-hydroxyvitamin D [25(OH)D]. Since 25(OH)D levels fluctuate with sun exposure, the mean of at least three measurements taken over the year was recorded. Likewise, the mean of three different determinations for haemoglobin, reticulocytes, calcium, phosphate, PTH and CRP was also calculated.

Patients underwent multislice spiral computed tomography to assess the degree of coronary calcifications, using a Somatom Sensation Cardiac 16 scanner (Siemens, Forchheim, Germany), and calcium score was calculated using a specific software (Syngo Ca-score; Siemens) in accordance with the Agatston system 56.

Peripheral blood specimens were collected before starting dialysis session to prevent any possible influence of dialyser on the cells, and discarding the first few drops to avoid needle-related contamination by resident endothelial cells.

Flow cytometry analysis of peripheral blood PACs and putative EPCs

Flow cytometry analysis was used to define and relatively quantify PACs and putative EPCs expressing (or not) VDR and OC. We followed a modified and improved version of our previous protocol [11], described in detail in the Supplementary Material.

Determination of circulating inflammation, cardiovascular and mineralization biomarkers

Luminex bead-based multiplex assays were carried out for determination of circulating interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α), fibroblast growth factor 23 (FGF23) and PTH. The detailed protocol is reported in the Supplementary Material.

Klotho levels were measured on plasma samples using a commercially available ELISA (Cusabio Biotech, Wuhan, China) following the manufacturer's instructions. Each sample was analysed in triplicate. The results, expressed as optical density, were interpolated on a standard curve generated from samples of known concentration (provided in the kit).

Statistical analysis

The dialysis patients were classified into two groups based on VDRA therapy (untreated and treated) and compared with healthy controls for demographic, clinical, biochemical and laboratory parameters using Kruskal–Wallis test for continuous variables and chi-square tests for categorical variables, followed by *post hoc* pairwise comparisons. Data of EPC relative counts and biochemical markers were log-transformed to achieve a normal distribution (after adding 1 to avoid negative values). Multiple linear regression analysis was used to evaluate the relationship between the relative counts of each cell subset and therapy, after adjusting for laboratory variables. A bootstrap procedure was applied to derive robust estimates of the standard errors and confidence intervals for the regression coefficients. The level of significance was set at 0.05. Statistical analyses were carried out using the 'Statistical package for the social sciences' (IBM SPSS Statistics, Version 20.0; SPSS, Inc., Chicago, IL, USA).

Results

Characteristics of subjects enrolled in the study

The demographic, clinical and biochemical characteristics of the population are summarized in Table 2. We analysed 23 controls, 18 haemodialysis patients not treated with VDRAs and 35 treated with VDRAs, 17 of them receiving between 1.25 and 1.75 μg/week oral calcitriol, and 18 between 10 and 15 μg/week IV paricalcitol. Moreover, among VDRA-treated patients, 20 patients were administrated calcium-free phosphate binders and 13 a combination of calcium-based and calcium-free phosphate binders. In the no-VDRA treatment group, all patients received a combination of calcium-based and calcium-free phosphate binders.

No participant reported a previous history of heart diseases, cerebrovascular atherosclerotic disease or peripheral vascular conditions. Among dialysis patients, 13 were diabetics, 6 were in the no-VDRA treatment group and 7 were in the VDRA treatment group. As expected, haemoglobin, low-density lipoprotein-cholesterol, calcium, albumin, ALP, iron and 25(OH)D levels were higher in the healthy subjects than in dialysis patients, and reticulocytes, serum creatinine and PTH values were lower. *Post hoc* pairwise comparisons revealed that VDRA-treated patients had higher albumin, iron and PTH levels than the untreated ones. No parameter differed significantly between patients treated with calcitriol or paricalcitriol, except PTH, which was higher in calcitriol-treated patients.

Quantitative and phenotypical assessment of endothelial progenitors

The relative counts (±standard deviation) of PACs and putative EPCs (respectively CD133+ or CD133−), expressing (or not) VDR and OC were determined by flow cytometry analysis. Both PACs and putative EPCs expressing VDR were significantly lower in healthy controls than in haemodialysis patients, either if CD133+ (VDR+ PACs: 12.70 ± 9.29 versus 21.75 ± 15.53, $P = 0.011$), or if CD133− (VDR+ putative EPCs: 11.78 ± 9.88 versus 21.96 ± 18.25, $P = 0.014$) (Figure 1A). A similar result was found in cell subsets not expressing VDR (VDR− PACs: 12.78 ± 9.22 versus 21.75 ± 15.53, $P = 0.009$; VDR− putative EPCs: 12.39 ± 10.25 versus 22.23 ± 18.26, $P = 0.014$). When haemodialysis patients were stratified according to the presence or absence of VDRA treatment, PACs positive for VDR relative counts were similar between healthy subjects and untreated haemodialysis patients, but significantly lower ($P < 0.05$) than in VDRA-treated ones (VDR+ PACs: 12.70 ± 9.29 versus 15.73 ± 10.43 versus 24.13 ± 15.57, $P = 0.008$). The relative count of putative EPCs positive for VDR was significantly lower in healthy subjects ($P < 0.05$) compared

Table 2. Demographic, clinical and biochemical parameters of the study groups

	(A) Healthy subjects ($n = 23$)	(B) VDRA-untreated patients ($n = 18$)	(C) VDRA-treated patients ($n = 18$)	P-value	*Post hoc* comparison
Gender (% female)	34.8	38.9	31.4	ns	\
Age (years)	60.4 ± 9.2	62.5 ± 15.2	60.3 ± 12.0	ns	\
Dialysis vintage (months)	\	20.5 ± 20.1	25.9 ± 33.4	ns	\
CKD duration (months)	\	75.6 ± 83.0	64.5 ± 65.1	ns	\
Diabetes (% of diabetics)	0.0	33.3	20.0	0.016	A < B, C
CTA (HU)	\	1062.3 ± 1386.8	411.7 ± 644.0	ns	\
Calcium score, Agatston units	\	1025.7 ± 1342.5	477.2 ± 794.6	ns	\
Erythropoietin dosage (UI)	\	15 333.3 ± 7584.4	15 222.2 ± 8556.1	ns	\
Haemoglobin (g/dL)	14.2 ± 1.3	10.9 ± 1.0	10.7 ± 1.2	<0.001	A > B, C
Reticulocytes (%)	1.1 ± 0.3	2.1 ± 0.6	1.9 ± 0.7	<0.001	A < B, C
sCreat (mg/dL)	0.8 ± 0.2	9.1 ± 3.6	9.8 ± 3.0	<0.001	A < B, C
HDL-cholesterol (mg/dL)	43.6 ± 8.6	37.5 ± 15.1	42.9 ± 14.0	ns	\
LDL-cholesterol (mg/dL)	119.4 ± 13.9	82.2 ± 35.4	90.0 ± 33.0	<0.001	A > B, C
Calcium (mg/dL)	9.1 ± 0.4	8.7 ± 0.6	8.8 ± 0.6	<0.001	A > B, C
Phosphate (mg/dL)	4.6 ± 1.0	5.5 ± 1.6	5.2 ± 1.3	ns	\
Albumin (g/dL)	4.7 ± 0.5	3.7 ± 0.3	4.1 ± 0.3	<0.001	A > C > B
ALP (UL)	107 ± 16.2	73.9 ± 37.0	91.6 ± 68.3	<0.001	A > B, C
Iron (mg/dL)	81.4 ± 36.9	43.3 ± 24.4	54.7 ± 21.4	<0.001	A > C > B
Ferritin (ng/dL)	143.5 ± 57.8	320.7 ± 307.4	216.7 ± 224.3	ns	\
PTH (pg/dL)	47.9 ± 18.4	189.4 ± 128.5	337.5 ± 191.6	<0.001	A <B < C
CRP (mg/dL)	0.8 ± 0.5	0.8 ± 1.0	0.8 ± 0.5	<0.001	\
BAP (mg/dL)	14.4 ± 8.3	14.8 ± 8.0	20.2 ± 15.8	ns	\
25(OH)D (pg/dL)	46.4 ± 21.5	17.7 ± 10.9	16.5 ± 9.5	<0.001	A > B, C
FGF23 (pg/mL)[a]	1.4 ± 0.3	3.1 ± 0.7	2.9 ± 0.7	<0.001	A < B, C
Klotho (pg/mL)[a]	0.1 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	<0.001	A < B, C

Categorical variables are presented as percentages, continuous variables as mean ± standard deviation. For haemoglobin, reticulocytes, calcium, phosphate, PTH, CRP and 25(OH)D the mean ± standard deviation of three measurements is reported (P-value: chi-square test/Kruskal–Wallis test; *post hoc* comparison: Kruskal–Wallis test). sCreat, serum creatinine; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ns, not significant.
[a]Indicates variables that were log-transformed.

with haemodialysis patients, with no differences between the VDRA-untreated and VDRA-treated groups (VDR+ PACs: 11.78 ± 9.88 versus 22.33 ± 11.40 versus 21.82 ± 17.02, P = 0.037) (Figure 1B). This finding was mirrored in VDR-negative cell phenotypes (VDR− PACs: 12.78 ± 9.22 versus 15.73 ± 10.43 versus 24.13 ± 15.57, P = 0.009; VDR− putative EPCs: 12.39 ± 10.25 versus 22.33 ± 11.40 versus 22.08 ± 17.04, P = 0.049).

OC-stained progenitors were also quantified in all subjects. Similar to VDR, the relative counts of PACs and putative EPCs expressing OC were significantly lower in healthy subjects than in haemodialysis patients (OC+ PACs: 12.57 ± 8.51 versus 20.23 ± 14.15, P = 0.018; OC+ putative EPCs: 11.70 ± 7.34 versus 20.32 ± 16.04, P = 0.016) (Figure 1C). Likewise, percentages of both cell subsets not expressing OC were lower in controls compared with dialysis patients (OC− PACs: 12.83 ± 8.45 versus 20.42 ± 14.33, P = 0.017; OC− putative EPCs: 12.91 ± 7.93 versus 21.11 ± 16.54, P = 0.022). Analysing VDRA treatment subgroups, the PACs positive for OC did not differ between healthy subjects and untreated patients (P > 0.05), but they were significantly lower (P < 0.05) than VDRA-treated ones (phenotype OC+ PACs: 12.57 ± 8.51 versus 14.47 ± 8.88 versus 22.50 ± 15.05, P = 0.030). Conversely, putative EPCs expressing OC were lower in healthy controls than in haemodialysis patients (P < 0.05), with no significant differences between untreated versus VDRA-treated patients (OC+ putative EPCs: 11.70 ± 7.34 versus 19.60 ± 8.78 versus 20.61 ± 16.13, P = ns) (Figure 1D). Also in OC− subsets, we found that those expressing CD133 (PACs) did not differ between healthy subjects and untreated patients (P > 0.05), but were significantly lower (P < 0.05) than in VDRA-treated ones (OC− PACs: 12.83 ± 8.45 versus 14.53 ± 8.90 versus 22.74 ± 15.23, P = 0.033). In contrast, putative EPCs negative for OC were lower in healthy controls than in dialysis patients (P < 0.05), with no significant differences between untreated versus VDRA-treated patients (OC− putative EPCs: 12.91 ± 7.93 versus 22.67 ± 9.07 versus 21.29 ± 16.80; phosphate and = ns). When the comparisons between the groups were adjusted for phosphate and PTH in linear regression models, these results were confirmed. However, when comparisons were adjusted for FGF23, no difference among groups was found, except for VDR+ PACs, which proved to be higher in both untreated and VDRA-treated patients compared with healthy subjects.

For all the cell subpopulations analysed here, no differences were found between patients treated with calcitriol or paricalcitriol (data not shown).

Correlation between VDR and OC expression in PACs and putative EPCs

Since in our flow cytometry experimental design fluorescent antibodies against VDR and OC antibodies were both labelled with FITC (fluorescein isothiocyanate), we could not examine the concomitant expression of the two molecules on EPCs. Therefore, linear regression analysis was used to determine positive cytometric events for PACs and putative EPCs expressing VDR or OC. As detailed in the Supplementary Figures S1 and S2, for both populations PACs and putative EPCs, significant positive correlations were found between cells expressing VDR

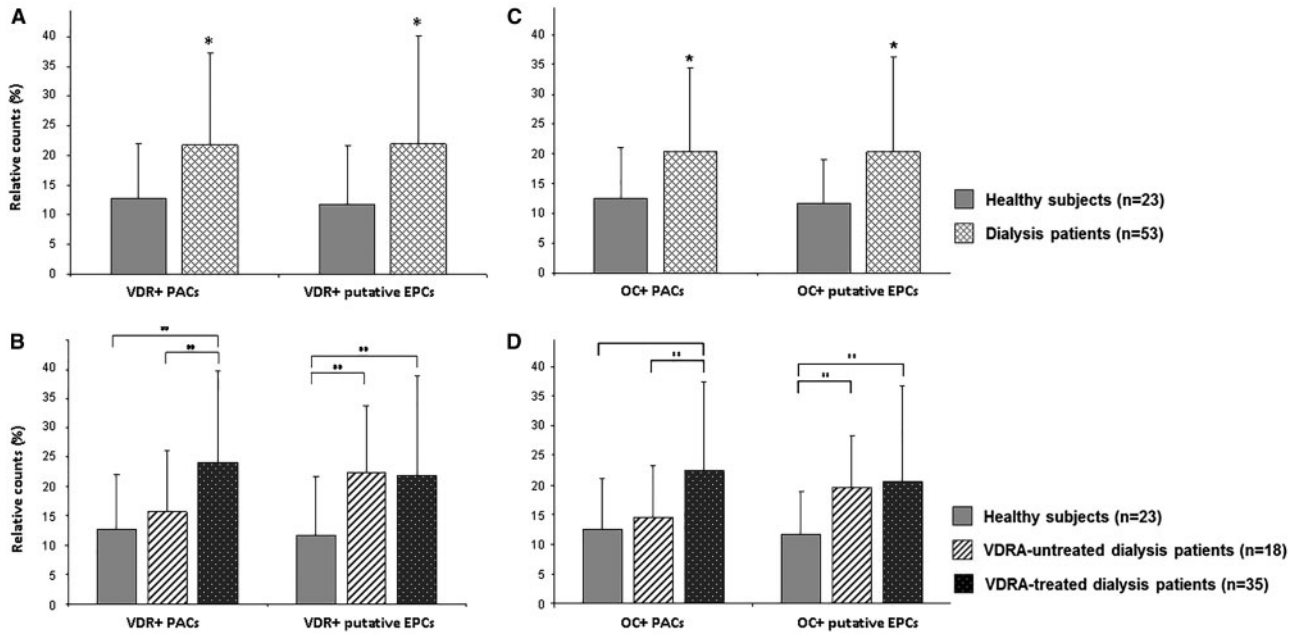

Fig. 1. (A and C) Relative counts of PACs (left) and putative EPCs (right) expressing VDR in the healthy subjects (gray bars) and dialysis patients (checked bars). Results are expressed as mean ± standard deviation and are calculated per 2×10^5 events measured (*$P < 0.05$). (B and D) Relative counts of PACs (left) and putative EPCs (right) expressing osteocalcin VDR in the healthy subjects (gray bars), VDRA-untreated dialysis patients (slashed bars) and VDRA-treated dialysis patients (dotted bars). Results are expressed as mean ± standard deviation and are calculated per 2×10^5 events measured (**$P < 0.01$).

and those expressing OC in the healthy controls, in untreated patients and in VDRA-treated patients, with no differences between patients receiving calcitriol or paricalcitriol (data not shown).

Serum levels of circulating inflammation, cardiovascular and mineralization biomarkers

Concerning circulating biomarkers of inflammation, cardiovascular and mineralization in healthy controls, VDRA-untreated patients and VDRA-treated patients, Kruskal–Wallis nonparametric test showed that IL-6, TNF-α, PTH, FGF23 and klotho (log-transformed values) were significantly lower in healthy controls compared with both groups of dialysis patients (all $P < 0.001$), with no differences between untreated and VDRA-treated patients (Supplementary Figure S3).

Correlations of PACs and putative EPCs with biochemical parameters

Univariate analysis was used to find associations of biochemical parameters and circulating inflammation, cardiovascular and mineralization biomarkers with all the analysed cell subsets in the three groups. No significant correlations emerged in the healthy subjects.

In untreated patients, PACs expressing OC phenotype correlated positively with the average (mean of three measurements) calcium levels ($R = 0.474$, $P = 0.047$; Figure 2) and reticulocyte count ($R = -0.608$, $P = 0.007$; Supplementary Figure S4).

In VDRA-treated patients, we observed significant positive correlations between PACs expressing VDR and IL-6 levels ($R = 0.381$, $P = 0.024$; Supplementary Figure S5A), and putative EPCs expressing OC and the mean value of 25(OH)D measurements over the year ($R = 0.333$, $P = 0.050$; Supplementary Figure S5B).

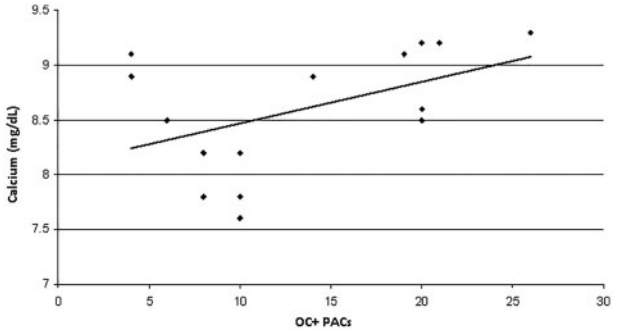

Fig. 2. Scatter plots and regression line showing the significant correlations in VDRA-untreated dialysis patients between the percentage of putative EPCs expressing OC and calcium levels determined as the mean of three measurements ($R = 0.474$, $P = 0.047$).

Discussion

Compared with the general population, patients with CKD have a reduced number and an impaired function of circulating EPCs and these abnormalities worsen with advancing disease [14]. The initiation of dialysis and a higher dialysis dose (Kt/V), in their turn, can improve the angiogenic function of EPCs [15]. To date, studies conducted on different phenotypes of putative EPCs in haemodialysis patients have mainly focused on the possible effects of ureamic toxins, dialysis modality and technique [16]. However, the results on quantitative changes in EPCs are variable, with two studies reporting slightly increased or similar cell levels in uraemic patients compared with normal renal function subjects [17, 18]. Despite the potential role of MBD and inflammation on bone health and bone marrow

microenvironment, poor data are currently available on the effect on EPCs of vitamin D and PTH on one hand, and cytokines and growth factors on the other.

Our first finding was the increased percentage of PACs and putative EPCs expressing VDR in haemodialysis patients compared with healthy subjects. To assess the effect of vitamin D replacement therapy, we divided the patients according to the treatment (or not) with VDRAs. Since in the subgroup receiving VDRAs we found no differences between patients treated with calcitriol or paricalcitriol for any of the parameters analysed here (except PTH), the patients were evaluated together, regardless of the administered drug. Specifically, the relative count of PACs expressing VDR were higher in VDRA-treated patients in comparison with both untreated patients and healthy controls. Nevertheless, considering putative EPCs expressing VDR, the differences between VDRA-treated and untreated patients disappeared, as both subgroups showed higher relative counts in comparison with healthy controls. Despite the limitations due to the cross-sectional nature of our study, this effect may be interpreted in the light of recent findings on the pathways involved in the release of HSCs (precursors of the EPCs) and EPCs from the bone marrow [19]. EPCs are mobilized by angiogenic homing factors to sites of neovascularization [19, 20]. Specific local tissue microenvironments (niches) in the bone marrow have been identified with the function of maintenance and regulation of HSCs/EPCs, keeping them (osteoblastic niche) for self-renewal and activating them (vascular niche) for proliferation and vascular repair [20]. Under normal and pathological conditions, there is a continuous egress out of the bone marrow of HSCs, including those committed to endothelial lineage: this process is termed mobilization [21]. Mobilization to bone marrow niches in the periphery and toward peripheral tissues is known as homing. Mobilization and homing are mirror processes depending on the interplay between several cytokines, growth factors and hormones (G-CSF (granulocyte-colony stimulating factor), IL-6, erythropoietin). The interaction between stromal cell-derived factor 1 (SDF1), also known as C-X-C motif chemokine 12 (CXCL12), and its receptor CXCR4 is the most important mechanism involved in retaining HSCs within the bone marrow and in HSCs/EPCs mobilization [22, 23]. Osteoblastic cells regulate the renewal of HSCs inside bone marrow niche since they express CXCL12, thereby binding HSCs that are primarily quiescent [24]. Therefore, any factor able to deplete osteoblasts and/or to reduce the expression of CXCL12 results in transmigration of HSCs and derived cell lineages into the vascular sinuses (peripheral circulation) [25].

PTH has been proven to control HSCs expansion in bone marrow niche by interaction with osteoblast PTH receptors, either by enhancing cytokine (IL-6 and IL-11) expression, or by increasing RANKL (receptor activator of nuclear factor kappa-B ligand) production, which in turn recruits osteoclasts causing mobilization of HSC into the circulation [26]. In haemodialysis patients the imbalance of osteoprotegerin (OPG)-RANKL system, whatever the cause may be, are involved in HSC egress from the bone marrow [8, 27].

PTH also stimulates HSCs mobilization and homing through modulation of the pivotal CXCL12/CXCR4 axis via endogenous release of G-CSF [22]. In our study, both untreated and VDRA-treated patients presented a cluster of factors, namely IL-6, PTH and recombinant erythropoietin (rhEPO), potentially able to enhance HSCs/EPCs mobilization and so the number of circulating EPC. The positive correlation found in treated patients between IL-6 and PACs expressing VDR is likely to be mediated by PTH, which showed higher levels in this subgroup of patients and is presumably responsible for the levels increased of IL-6 and the related mobilization of PACs from bone marrow. Alternatively inflammation, *per se*, could play a direct role. PTH also stimulates HSCs mobilization and homing through modulation of the pivotal CXCL12/CXCR4 axis via endogenous release of G-CSF [22].

An additional factor able to trigger a rise of PACs expressing VDR might be VDR activation [12, 28, 29]. This effect of VDRAs on EPC phenotypes feasibly follows the expression of protective genes thus inducing a longer survival [12]. The common impairment of endothelial function observed in late-stage CKD dialysis patients might represent a further explanation for this increase in circulating EPCs [29]. Güven *et al.* proposed that the EPC count might reflect the severity of endothelial damage, and express a continuous attempt to repair endothelial injury and restore its function [30]. Furthermore, the high percentage of PACs expressing VDR found in VDRA-treated patients could probably be ascribed to both VDRAs (pharmacological effect) and PTH-dependent upregulation of VDR and 1-α hydroxylase [31].

However, our results indicate that even after controlling for PTH levels, the percentage of PACs expressing VDR in VDRA-treated patients remains significantly higher than in healthy controls.

A second noticeable finding of this study was the significant increase in the percentage both PACs and putative EPCs expressing OC in uraemic patients versus healthy subjects. Indeed, analysing the haemodialysis patients according to the treatment with VDRAs, we noticed that the relative count of PACs expressing OC was higher only in the VDRAs-treatment subgroup, with no differences between healthy subjects and untreated patients. Moreover, in VDRA-untreated patients this specific subset was directly correlated with calcium levels. This result could be traced to an effect on HSCs mobilization/homing of calcium levels opposite to endosteal calcium concentration mediated by CaSR expressed on HSCs [32]. In contrast, the percentage of putative EPCs expressing OC did not differ between untreated and VDRA-treated patients. This cell subset also correlated positively with 25(OH)D levels in patients receiving VDRA therapy. OC is expressed/produced by osteoblastic cells and circulating osteoprogenitor cells of haematopoietic and mesenchymal origin, and its regulation involves a number of calcitropic hormones and growth factors including 1,25-dihydroxyvitamin D3, PTH, bone morphogenetic proteins, TNF-α and transforming growth factor β [31, 33]. Eghbali-Fatourechi *et al.* reported that OC is also expressed by circulating CD34+ progenitor cells [34]. PACs and putative EPCs are able to differentiate into osteoblasts, haematopoietic and endothelial cells *in vitro*, thereby implying some degree of overlap between cells staining for osteoblastic markers and those of the haematopoietic/endothelial lineage [5]. Therefore, the large amount of PACs and putative EPCs expressing OC found in VDRA-treated patients could be ascribed to a combined effect of 25(OH)D, VDRA therapy and, last but not least, PTH that, as expected, had higher levels in this subgroup of patients. It is conceivable that the action of these factors is fulfilled on both cell subsets, analysed in different districts (bone marrow and bloodstream) and at different times. The effect of PTH on OC expression may have been blunted by VDRA treatment. Moreover, VDR expression is associated with 25(OH)D levels and vitamin D therapy, so the strong correlation between VDR and OC expression, in both phenotypes, suggests also a possible link of OC with vitamin D status.

The above-mentioned differences across the three groups were confirmed when adjusted for phosphate and PTH in linear regression models. However, after adjustment for FGF23, no difference between the groups was found, except for VDR+ putative EPCs, that were higher in VDRA-treated and untreated patients compared with healthy subjects. Although the cross-sectional study design does not allow us to provide a clear-cut interpretation of this result, we propose as a tentative hypothesis that the inhibition of the synthesis of 1,25(OH)D by FGF23 plays relative a role. FGF23 and klotho are mutually and independently critical regulators of mineral metabolism. Epidemiological data have supported a causal role of the impairment of FGF23-klotho axis in the onset and progression of vascular calcification, although this issue is still under debate [35]. In contrast to previous studies, klotho levels were higher in dialysis patients, but perhaps this discrepancy may be due to differences in the protocols used for klotho assay [36].

The effect of rhEPO on EPC mobilization deserves a special mention. Erythropoietin is known to promote EPC proliferation, differentiation and adhesion, indeed erythropoietin-induced neovascularization is mediated through EPCs recruited from the bone marrow. Even if the dose of rhEPO in VDRA-treated and untreated patients was similar, a possible effect of rhEPO was indirectly confirmed in patients not treated with VDRAs by the significant association of the PACs expressing OC with reticulocyte count. This result diverges from the findings reported by Schlieper *et al.*, indicating haematocrit and reticulocyte counts as negative predictors of EPC numbers [37].

An interesting finding in our study was that, while PACs expressing OC were increased only in patients under VDRA treatment, putative EPC expressing OC were also high in untreated patients. Since the antigen CD133 is a stemness marker that is lost during EPC differentiation, taken together, these findings seem to indicate the acquisition of an osteogenic phenotype in the early stages of maturation process, with a later contribution given by the exposure to the uraemic milieu.

Regarding the role of age and gender, there were no age differences between the groups, or in terms of OC-expression between males and females. It is known that in the general population, the count of circulating OC-positive cells rises as a function of age [34]. Uraemic syndrome is the paradigmatic model of premature aging, caused by phosphate retention, decreased klotho expression/blood levels, accumulation of uraemic toxins triggering oxidative stress and inflammation-induced cellular senescence and stem cell exhaustion, factors that in turn may promote vascular disease and accelerated aging [38]. On a purely hypothetical basis, calcifying EPCs could represent another manifestation of uraemic premature aging. The presence in haemodialysis patients of EPCs with a osteogenic phenotype is relevant as several preclinical studies and preliminary clinical evidence indicate that EPCs home to sites of vascular damage and that OC expression on circulating EPCs is significantly associated with coronary artery disease, diabetes and valvular calcification. Whatever the triggers may be, this endothelial-to-procalcific shift of circulating cells has the potential to deliver a calcifying stimulus to the vasculature via cells that are naturally instructed to enter the vessel wall.

Our observations confirm that MBD disorders and inflammation (IL-6) may be related to adverse effects in CKD patients. Furthermore, if uraemia is set up as a potentially procalcifying milieu, we failed to demonstrate a protective effect by VDRA therapy. The role of vitamin D deficiency in cardiovascular disease is a relatively novel field of interest. Well-substantiated experimental data describe the regulatory effects of vitamin D on cardiovascular risk factors, such as diabetes mellitus, and on the renin-angiotensin system, along with the reasonable protection against vascular calcification. In contrast, vitamin D overdosing might induce hypercalcaemia, hyperphosphataemia and promote FGF23 expression and vascular calcification. The poor evidence on vitamin D therapy benefits currently prevents general recommendations on its application.

This study has some limitations, including the relatively small number of patients. Moreover, our results are based on cross-sectional data, and we therefore cannot draw any firm conclusions with respect to causality or change in the variables considered over time. A further weakness is represented by the lack of correlation between endothelial precursors and clinical/instrumental end points, but this was not part of the study design. Despite these drawbacks, our study suggests that CKD patients have an increased number of EPCs expressing an osteogenic phenotype, a factor of bone-vascular axis potentially relevant to vascular calcification pathogenesis, that may be modulated by and vitamin D therapy.

Funding statement

This research received no specific grant from any funding agency in the public or commercial sectors.

Supplementary data

Supplementary data are available online at http://ckj.oxfordjournals.org.

Conflict of interest statement

The results presented in this article have not been published previously in whole or part, except in abstract form. None of the authors has conflicts of interest to disclose.

References

1. Hyder JA, Allison MA, Wong N *et al.* Association of coronary artery and aortic calcium with lumbar bone density: the MESA Abdominal Aortic Calcium Study. *Am J Epidemiol* 2009; 169: 186–194
2. Nakamura S, Ishibashi-Ueda H, Niizuma S *et al.* Coronary calcification in patients with chronic kidney disease and coronary artery disease. *Clin J Am Soc Nephrol* 2009; 4: 1892–1900
3. Shanahan CM, Crouthamel MH, Kapustin A *et al.* Arterial calcification in chronic kidney disease: key roles for calcium and phosphate. *Circ Res* 2011; 109: 697–711
4. Fadini GP, Rattazzi M, Matsumoto T *et al.* Emerging role of circulating calcifying cells in the bone-vascular axis. *Circulation* 2012; 125: 2772–2781
5. Fadini GP, Losordo D, Dimmeler S. Critical reevaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use. *Circ Res* 2012; 110: 624–637
6. Richardson MR, Yoder MC. Endothelial progenitor cells: quo vadis? *J Mol Cell Cardiol* 2011; 50: 266–272
7. Rose JA, Erzurum S, Asosingh K. Biology and flow cytometry of proangiogenic hematopoietic progenitors cells. *Cytometry A* 2015; 87: 5–19
8. Mazzaferro S, Tartaglione L, Rotondi S *et al.* News on biomarkers in CKD-MBD. *Semin Nephrol* 2014; 34: 598–611
9. Gössl M, Mödder UI, Gulati R *et al.* Coronary endothelial dysfunction in humans is associated with coronary retention of

osteogenic endothelial progenitor cells. *Eur Heart J* 2010; 31: 2909–2914
10. Fadini GP, Albiero M, Menegazzo L *et al*. Procalcific phenotypic drift of circulating progenitor cells in type 2 diabetes with coronary artery disease. *Exp Diabetes Res* 2012; 2012: 921685
11. Cianciolo G, La Manna G, Cappuccilli ML *et al*. VDR expression on circulating endothelial progenitor cells in dialysis patients is modulated by 25(OH)D serum levels and calcitriol therapy. *Blood Purif* 2011; 32: 161–173
12. William-Ignarro S, Byrns R, Balestrieri ML *et al*. Therapeutic targeting of the stem cell niche in experimental hindlimb ischemia. *Nat Clin Pract Cardiovasc Med* 2008; 5: 571–579
13. Aguirre A, González A, Planell JA *et al*. Extracellular calcium modulates in vitro bone marrow-derived Flk-1+ CD34+ progenitor cell chemotaxis and differentiation through a calcium-sensing receptor. *Biochem Biophys Res Commun* 2010; 393: 156–161
14. Krieter DH, Fischer R, Merget K *et al*. Endothelial progenitor cells in patients on extracorporeal maintenance dialysis therapy. *Nephrol Dial Transplant* 2010; 25: 4023–4031
15. Choi JH, Kim KL, Huh W *et al*. Decreased number and impaired angiogenic function of endothelial progenitor cells in patients with chronic renal failure. *Arterioscler Thromb Vasc Biol* 2004; 24: 1246–1252
16. Ramirez R, Carracedo J, Merino A *et al*. Microinflammation induces endothelial damage in haemodialysis patients: the role of convective transport. *Kidney Int* 2007; 72: 108–113
17. Herbrig K, Pistrosch F, Oelschlaegel U *et al*. Increased total number but impaired migratory activity and adhesion of endothelial progenitor cells in patients on long-term haemodialysis. *Am J Kidney Dis* 2004; 44: 840–849
18. Steiner S, Schaller G, Puttinger H *et al*. History of cardiovascular disease is associated with endothelial progenitor cells in peritoneal dialysis patients. *Am J Kidney Dis* 2005; 46: 520–528
19. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature* 2014; 505: 327–334
20. Yamaguchi J, Kusano KF, Masuo O *et al*. Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. *Circulation* 2003; 107: 1322–1328
21. Brunner S, Zaruba MM, Huber B *et al*. Parathyroid hormone effectively induces mobilization of progenitor cells without depletion of bone marrow. *Exp Hematol* 2008; 36: 1157–1166
22. Huber BC, Grabmaier U, Brunner S. Impact of parathyroid hormone on bone marrow-derived stem cell mobilization and migration. *World J Stem Cells* 2014; 6: 637–643
23. Petit I, Szyper-Kravitz M, Nagler A *et al*. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat Immunol* 2002; 3: 687–694
24. Calvi LM, Adams GB, Weibrecht KW *et al*. Diabetic stem-cell "mobilopathy". *Nature* 2003; 425: 841–846
25. Di Persio JF. Diabetic stem-cell "mobilopathy". *N Engl J Med* 2011; 365: 2536–2538
26. Porter RL, Calvi LM. Communications between bone cells and hematopoietic stem cells. *Arch Biochem Biophys* 2008; 473: 193–200
27. Cianciolo G, La Manna G, Donati G *et al*. Effects of unfractioned heparin and low-molecular-weight heparin on osteoprotegerin and RANKL plasma levels in haemodialysis patients. *Nephrol Dial Transplant* 2011; 26: 646–652
28. Yiu YF, Chan YH, Yiu KH *et al*. Vitamin D deficiency is associated with depletion of circulating endothelial progenitor cells and endothelial dysfunction in patients with type 2 diabetes. *J Clin Endocrinol Metab* 2011; 96: E830–E835
29. Moody WE, Edwards NC, Madhani M *et al*. Endothelial dysfunction and cardiovascular disease in early-stage chronic kidney disease: cause or association? *Atherosclerosis* 2012; 223: 86–94
30. Güven H, Shepherd RM, Bach RG *et al*. The number of endothelial progenitor cell colonies in the blood is increased in patients with angiographically significant coronary artery disease. *J Am Coll Cardiol* 2006; 48: 1579–1587
31. Somjen D, Knoll E, Sharon O *et al*. Calciotrophic hormones and hyperglycemia modulate vitamin D receptor and 25 hydroxyy vitamin D 1-á hydroxylase mRNA expression in human vascular smooth muscle cells. *J Steroid Biochem Mol Biol* 2015; 148: 210–213
32. Drüeke TB. Haematopoietic stem cells–role of calcium-sensing receptor in bone marrow homing. *Nephrol Dial Transplant* 2006; 21: 2072–2074
33. Jiang D, Franceschi RT, Boules H *et al*. Parathyroid hormone induction of the OC gene. Requirement for an osteoblast-specific element 1 sequence in the promoter and involvement of multiple-signaling pathways. *J Biol Chem* 2004; 279: 5329–5337
34. Eghbali-Fatourechi GZ, Mödder UI, Charatcharoenwitthaya N *et al*. Characterization of circulating osteoblast lineage cells in humans. *Bone* 2007; 40: 1370–1377
35. Olauson H, Vervloet MG, Cozzolino M *et al*. New insights into the FGF23-Klotho axis. *Semin Nephrol* 2014; 34: 586–597
36. Nowak A, Friedrich B, Artunc F *et al*. Prognostic value and link to atrial fibrillation of soluble Klotho and FGF23 in haemodialysis patients. *PLoS One* 2014; 9: e100688
37. Schlieper G, Hristov M, Brandenburg V *et al*. Predictors of low circulating endothelial progenitor cell numbers in haemodialysis patients. *Nephrol Dial Transplant* 2008; 23: 2611–2618
38. Stenvinkel P, Larsson TE. Chronic kidney disease: a clinical model of premature aging. *Am J Kidney Dis* 2013; 62: 339–351