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Original Article

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Association between phosphate removal and markers of bone turnover in haemodialysis patients

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Abstract

Background. As the main mineral reservoir, bone acts as a calcium (Ca) and phosphate buffering system. Accordingly, phosphate removal by haemodialysis (HD) might be theoretically influenced by bone turnover, as well as by the interaction of regulatory molecules, such as PTH and osteoprotegerin (OPG). The present study investigated the relationship between these variables and phosphate removal by HD.

Methods. Blood samples for serum Ca, phosphate, bicarbonate, intact PTH, PTH (1–84), bone alkaline phosphatase, tartrate-resistant acid phosphatase 5b, OPG and receptor activator of nuclear factor-κB ligand (RANKL) were obtained in 28 HD patients. Phosphate removal was measured by a continuous collection of the dialysate.

Results. Pre-dialysis serum phosphate concentration is the critical factor in determining dialytic phosphate removal. However, multiple regression analysis reveals that phosphate removal is better explained by a combination of factors than by phosphate concentration alone. In this model, the PTH/OPG ratio is an additional positive factor, whereas age and vitamin D treatment are negative factors. Patients with pre-HD bicarbonate higher than 20 mEq/l had higher serum phosphate and, accordingly, higher phosphate removal; of interest, these individuals also have significant differences in RANKL/OPG. Mean (SD) OPG levels were significantly higher than that in the healthy population (16.2 (12.5) pmol/l; these values correlated with age ($r = 0.4$, $P < 0.04$). Mean serum RANKL (1.03 (1.02) pmol/l) was within the range of normal individuals.

Conclusions. Dialytic phosphate removal has a crucial, direct relationship with pre-HD plasma phosphate levels. However, the phenomenon of phosphate removal is more precisely explained using a more complex relationship, defined by the interaction between serum phosphate, PTH/OPG, age and vitamin D administration. Serum RANKL levels are first reported in HD patients, and are not different from the normal population.

Keywords: Bone turnover; haemodialysis; osteoprotegerin; phosphate removal; PTH; RANKL

Introduction

Disturbances of phosphate in chronic renal failure are not only related to bone disease, but also contribute significantly to the high cardiovascular mortality in the dialysed population. Hyperphosphataemia and an increased Ca × P product have been directly linked to increased mortality in a large number of haemodialysis (HD) patients, therefore emphasizing the importance of adequately controlling hyperphosphataemia [1,2].

Control of phosphate in HD patients depends on dietary restriction, binder administration and dialysis therapy [3]. However, the average dialytic phosphate removal is insufficient and many individuals on HD are in positive balance. During a 4 h HD session, there is a first phase of rapid reduction of plasma phosphate, typically lasting 60–90 min. After this initial phase, the phosphate diffusion gradient is decreased and the rate of elimination diminishes [4]. Accordingly, the total amount of phosphate removed in the first 90 min exceeds the amount removed in the time left; the critical rate-limiting factor in this last phase is phosphate transfer from low interchangable compartments to the extracellular fluid. After dialysis is completed,

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a new balance is reached in several hours, during the interdialytic period [5]. This particular kinetics is due to the existence of multiple phosphate pools, which balance with plasma by incompletely defined equilibrium constants; in addition, the rate of phosphate release from non-dialysed pools is quite variable and may change several fold during HD.

Our recent data in a smaller group of patients have suggested that phosphate removal depends on the initial plasma phosphate; however, higher phosphate elimination appeared to be associated with higher plasma intact parathyroid hormone (PTHi) [6]. At first glance, the interpretation of this finding would be that higher PTH induces increased bone turnover, with the subsequent phosphate release from bone. Accordingly, we primarily hypothesized that part of the so-called 'intracellular' efflux of phosphate during HD could be kinetically described as a rather labile bone pool of phosphate and that the PTH-regulated activity of this pool might be a determinant of phosphate availability for HD. However, PTHi alone does not provide sufficient information to accurately characterize bone metabolism; therefore, it is predictable that correlations would be more consistent when other markers of bone activity are incorporated into the analysis. These variables include the tartrate-resistant acid phosphatase 5b (TRACP)-5b as a marker for osteoclastic activity and serum bone alkaline phosphatase (bAP), as a marker of bone formation and turnover [7]. Moreover, an improved diagnosis of renal osteodystrophy could be attained with the introduction of second generation immunometric assays for PTH that measure full-length, biologically active whole PTH (1–84) [8,9].

In addition, local factors involved in the process of bone turnover and in mediating the effect of PTH have been identified, e.g. cytokine systems regulating osteoclastogenesis. In this regard, osteoprotegerin (OPG), receptor activator of nuclear factor (NF)-κB (RANK) and RANK ligand (RANKL) constitute a complex mediator system involved in the regulation of the resorption process in bone. Osteoclast precursors express RANK, a membrane-bound receptor, which recognizes RANKL through cell–cell interaction with osteoblast/stromal cells. This interaction enables osteoclast precursors to differentiate in the presence of macrophage colony-stimulating factor. OPG recognizes RANKL, and this decoy receptor blocks the interaction between RANK and RANKL, leading to inhibition of osteoclast differentiation and activation. In bone tissue, OPG and RANKL are expressed by osteoblasts and the ratio of these products may modulate the ability of these cells to stimulate osteoclast differentiation/activity, as well as the rate of bone resorption. Recent studies have demonstrated that osteotropic factors and hormones such as PTH, vitamin D or TNF-α upregulate RANKL expression in osteoblast/stromal cells. In addition, OPG expression is downregulated by prostaglandin E2, and is upregulated by oestrogens. RANK expression has not yet been extensively studied. Therefore, a number of osteotrophic factors and hormones may modulate bone resorption via this common final pathway [10].

Relevant studies have been recently published on the role of OPG in renal osteodystrophy and its involvement in PTH resistance. Collectively, they show that HD patients have higher OPG levels than healthy individuals; furthermore, OPG is not removed by HD and its levels are age-related, as in the normal population [11,12]. However, in individuals with chronic renal failure, no consistent correlations between OPG levels and PTH, Ca or phosphate have been found [13,14,15]. To our knowledge, no data are available concerning circulating RANKL in HD patients. In the same regard, no data are available about the balance in RANKL/OPG and their possible correlations; these variables are potentially important for a more accurate definition of bone activity, indicating the equilibrium between pro- and anti-resorptive pathways that determine bone activity.

In light of these considerations, the hypothesis was raised that bone metabolism and hormone-cytokine parameters involved in phosphate distribution are related to the rate of phosphate removal by HD. Accordingly, a study was designed to analyse bone and phosphate turnover as complex, multiregulated phenomena. Dialytic elimination of phosphate was directly measured, in parallel with biochemical determinations aimed at differentiating bone activity (bAP, TRACP-5b, PTHi and PTH (1–84)). Measurements of cytokines putatively involved in the regulation of bone resorption, namely, OPG and RANKL, were done simultaneously, to further characterize each patient's situation.

Subjects and methods

Study design and patients

A prospective study was conducted in two dialysis units of a university hospital. Patients examined included 28 clinically stable individuals (18 male/10 female, age range 37–85 years, median 66 (13) years). All of them had been on HD for more than 6 months [mean 30 (39.1) months], and residual diuresis was less than 200 ml/day in all the cases. The Institutional Board approved the protocol and written informed consent was obtained from all the participants. The aetiology of the renal disease was: glomerulonephritis ($n=5$), vascular ($n=7$), unknown ($n=6$), diabetes ($n=4$) and other ($n=6$). All the patients received oral phosphate binders, i.e. calcium carbonate, calcium acetate, sevelamer or aluminium hydroxide, which were unchanged during the study. None of them had clinical or biochemical markers of liver disease, and serum GGT levels were normal. Nine patients were on treatment with oral or intravenous vitamin D and the range of serum PTH levels was wide.

Dialysis characteristics and biochemical determinations

Studies were performed in a mid-week HD session, using a $2\,m^2$ dialyser (12 synthetic and 16 cellulose di- or

triacetate membranes), with identical conditions of blood (350 ml/min) and dialysate flow (500 ml/min) and duration (4 h). All the patients had a Kt/V between 1.3 and 1.5. Dialysate composition was: Na^+ 139 mEq/l, K^+ 1.5 mEq/l, Ca^{++} 3 mEq/l, glucose 1.5 g/l and HCO_3^- only 37 mEq/l. No food was allowed for 2 h before nor during the dialysis session.

Blood was drawn at the beginning of HD for the following determinations: Blood urea nitrogen (BUN), calcium, phosphate, total alkaline phosphatase (tAP) and bicarbonate (SMAC-20 autoanalyser), PTHi, PTH (1–84), TRACP, OPG and RANKL. A blood sample at the end of the HD session was drawn for BUN determination.

Also, fractional collection of the total effluent volume of dialysate was continuously performed by a sampling pump incorporated within the hydraulic system of the dialysis machines [16] (Quantiscan Hospal, Madrid). This method enables reliable collection of a representative sample of the total waste dialysate. The total waste dialysate volume and collection time are displayed on the monitoring screen. Multiplication of the solute's mean concentration (C_m) by the total waste dialysate volume (V_d) gives the total amount of this solute removed (TM): $TM = C_m \times V_d$. In our own setting-up studies, measurement of phosphate in this type of sampling accurately correlated with that obtained by the complete collection of the dialysate ($r = 0.94$, $P < 0.001$, $n = 5$) and with measurements done with a home-made collection system using a reverse infusion pump. Calibration of the systems was checked weekly.

Phosphate and urea concentrations were measured in the dialysate to calculate mass removal. Serum phosphate was measured by a Roche Modular DDPP autoanalyser. Inorganic phosphorous forms a complex with ammonium molibdate, which is quantified by UV absorbance at 340 nm. Inter- and intra-assay coefficients of variation of the method were 0.9 and 1.4, respectively. Sensitivity of the method was 0.3 mg/dl. Phosphate effluent concentration was within the range of determination accuracy. The Kt/V was calculated by Daugirdas 2nd generation equation.

PTH assays

The PTH Scantibodies duo-PTH immunoassay (Scantibodies Laboratories Inc, Santee, CA) was employed. It contained two PTH assays for total (or intact PTH) and whole PTH [PTH (1–84)]. The assay of PTHi is able to detect PTH (1–84) and the PTH (7–84) non-biologically active PTH fragments. However, the whole PTH assay detects only the true intact PTH (1–84) molecule. Inter- and intra-assay coefficients of variation of the method were <8 and <5%, respectively. Sensitivity of the method was 1 pg/ml for whole PTH and 1.23 pg/ml for intact PTH. Non-PTH (1–84) (PTHc) was quantified by subtracting the values of whole PTH from those of the intact PTH.

Biochemical markers of bone metabolism

(i) Tartrate-resistant acid phosphatase for 5b (TRACP-5b): BoneTRAP® solid-phase immunofixed enzyme activity assay (Suomen Bioanalytiikka Oy, Oulu, Finland) was used for the determination of TRACP-5b. The inter- and intra-assay variation were <8 and <6%, respectively. Analytical sensitivity of the assay is 0.06 U/l.

(ii) Bone alkaline phosphatase (bAP): The Tandem®-R Ostase® solid-phase, two-site immunoradiometric assay (Beckman Coulter TM) was used for the quantitative measurement of bAP. The inter- and intra-assay coefficients of variation were <8.1 and <6.8%, respectively. The minimum detectable concentration was 2 µg/l.

(iii) Osteoprotegerin (OPG): The OPG was measured by Biomedica Gruppe (Wien, Austria) enzyme immunoassay, which uses two highly specific antibodies against human OPG. Intra- and inter-assay variation coefficients were <10%. Detection limit was 0.14 pmol/l.

(iv) Receptor activator of nuclear factor (NF)-κB ligand (RANKL): The RANKL was measured by Biomedica Gruppe (Wien, Austria) enzyme immunoassay, which determines soluble RANKL directly in biologic fluids. Inter- and intra-assay coefficients of variation were <9 and <5%, respectively. Sensitivity was 0.08 pmol/l.

Statistics

Results are expressed as mean (SD). The Student's *t*-test or ANOVA were used to compare continuous variables (expressed as mean SD). The Bonferroni restriction test was applied to each test of significance and to *post hoc* multiple comparisons. Simple linear regression was applied when appropriate and Pearson's coefficients of correlation were obtained between biochemical variables. A multivariate model was employed, that used a backward stepwise method. Variables to detect predictive factors on phosphate removal in the multivariate model were included on the basis of the univariate results. Adjusted parameters (beta) and their 95 percent confidence intervals (95% CI) were calculated. Further analysis was done using some relevant variables. The median value was taken as a cut-point reference. The null hypothesis was rejected in each statistical test when P was <0.05. Analysis was performed using the Windows SPSS (version 10.0) software.

Results

Average values of the variables are shown in Table 1. As regards to phosphate removal, Pearson's correlation coefficients for the entire cohort were statistically significant for baseline serum phosphate ($r = 0.59$, $P < 0.002$), PTHi/OPG ratio ($r = 0.52$, $P < 0.008$), RANKL/OPG ratio ($r = 0.46$, $P < 0.02$), serum bicarbonate ($r = -0.58$, $P < 0.01$), OPG ($r = -0.51$, $P < 0.009$) and age ($r = -0.43$, $P < 0.03$). No correlations were found between phosphate removal and other measured parameters, including Kt/V and nPCR.

In an effort to find the variables that were more informative for phosphate removal, we studied a set of specific correlations. Serum phosphate was related with RANKL/OPG ratio ($r = 0.5$, $P < 0.01$). OPG was positively related with age ($r = 0.39$, $P < 0.04$) and negatively with PTHi/OPG ratio ($r = -0.4$, $P < 0.03$). Age correlated positively with bicarbonate ($r = 0.49$, $P < 0.04$), and negatively with PTHi/OPG ratio ($r = -0.54$, $P < 0.003$) and RANKL/OPG ratio ($r = -0.55$, $P < 0.003$). One additional relationship

was found between PTHi/OPG and RANKL/OPG ($r = 0.4$, $P < 0.03$).

A stepwise multiple linear regression analysis was performed to determine the factors that predict phosphate removal. The paradigm included the variables that had shown significant influence in the univariate analysis and the use of vitamin D. Differences in phosphate removal were satisfactorily explained by a model based in serum phosphorus, PTHi/OPG, use of vitamin D and age (Table 2). In terms of putative implications in parameters with influence on bone turnover, PTHi/OPG ratio was positively related to RANKL/OPG ($r = 0.4$, $P < 0.03$), TRACP-5b ($r = 0.49$, $P < 0.01$), bAP ($r = 0.51$, $P < 0.007$) and negatively related with age ($r = -0.54$, $P < 0.003$).

Further comparisons of the biochemical parameters were performed with the purpose of detecting clues which might have not been readily apparent in a first-round analysis. Accordingly, phosphate removal was compared in high turnover ($n = 6$) (defined by PTHi >450 pg/ml and bAP >20 μg/l) *vs* low turnover ($n = 9$) (PTHi <150 pg/ml and bAP <15 μg/l). Both groups were classified using extreme biochemical values, for discriminating clearly defined patterns of bone disease; the differences between high and low bone turnover patients were not statistically significant (Table 3).

The finding that the PTH/OPG ratio was an independent predictive value led us to further study its possible influence on phosphate elimination. For this purpose, the group was separated in tertiles, with nine patients with PTH/OPG lesser than 10.7 and nine patients above 40. The results of this analysis are shown in Table 4, and reveal that the group with higher PTH/OPG ratio had higher phosphate elimination; however, this elimination coincided with a more elevated plasma phosphate.

When the patients were separated in tertiles according to age, and individuals younger than 60 years ($n = 10$) were compared with those older than 70 years ($n = 8$), younger individuals had higher initial serum phosphate (6.5 (2.5) *vs* 4.7 (0.9) mg/dl, $P < 0.04$), phosphate removal (1162.5 (348.9) *vs* 866.7 (118.9) mg, $P < 0.05$), RANKL/OPG ratio (0.14 (0.12) *vs* 0.03 (0.03), $P < 0.006$) and a lower OPG (11.5 (11.2) *vs* 20.6 (12.4) pmol/l, $P < 0.05$). Further, correlation analysis showed that OPG was directly related to age ($r = 0.4$, $P < 0.04$). When patients were examined according to pre-HD bicarbonate (higher or lower than 20 mEq/l), those with lower bicarbonate had higher phosphate removal and serum phosphate, were significantly younger and had significant differences in RANKL/OPG (Table 4). Finally, further comparisons were performed based on vitamin D administration; this analysis indicated that vitamin D-treated patients were older and had higher OPG levels than non-treated patients (Table 4).

Table 1. Values of biochemical parameters of all patients

	Mean (SD)
Age (years)	64.6 (13)
Serum Ca (mg/dl)	9.4 (0.8)
Serum phosphate (mg/dl)	5.4 (1.6)
Serum bicarbonate (mEq/l)	20.6 (3.1)
PTHi (pg/ml)	429.7 (379.6)
PTH (1–84) (pg/ml)	353 (350)
PTHc (pg/ml)	76.7 (79.8)
tAP (U/l)	138.7 (130.1)
bAP (μg/l)	36.7 (43.1)
TRACP-5b (U/l)	4.6 (2.5)
RANKL (pmol/l)	1.03 (1.02)
OPG (pmol/l)	16.2 (12.5)
PTHi/OPG	41.7 (49)
RANKL/OPG	0.08 (0.1)
Phosphate removal (mg/session)	1089 (287.1)
Kt/V	1.4 (0.1)
nPCR	1.1 (0.1)

tAP: total alkaline phosphatase; bAP: bone alkaline phosphatase; PTHi: intact PTH; TRACP-5b: tartrate-resistant acid phosphatase 5b; OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor (NF)-κB ligand.
To convert serum calcium from mg/dl to mEq/l, multiply by 0.25, serum phosphate from mg/dl to mmol/l, multiply by 0.32.

Table 3. Comparison of phosphate removal between patients with adynamic bone disease (ABD) and secondary hyperparathyroidism (SPTH)

	ABD ($n = 6$)	SPTH ($n = 9$)	P
Phosphate removal (mg)	1175 (317.2)	1124 (331.5)	NS
Serum phosphate (mg/dl)	5.3 (1.2)	5 (1.5)	NS
PTHi (pg/ml)	68.3 (42)	947.6 (249.2)	0.000
PTH (1–84) (pg/ml)	48.5 (28)	810.1 (308.2)	0.000
PTHc (pg/ml)	19.8 (16.9)	137.5 (118.9)	0.03
PTH (1–84)/PTHc	6.7 (7.3)	20.7 (23.9)	NS
PTH/OPG	10.2 (8.7)	75.2 (52.3)	0.01
TRACP-5b (U/l)	2.8 (1.1)	8.3 (2.1)	0.000
bAP (μg/l)	12.8 (1.4)	84.9 (57.9)	0.01

bAP: bone alkaline phosphatase; PTHi: intact PTH; TRACP-5b: tartrate-resistant acid phosphatase 5b. NS: not significant.
To convert serum phosphate from mg/dl to mmol/l, multiply by 0.32.

Table 2. Multiple linear regression analysis to detect predictive factors on phosphate removal ($r^2 = 0.75$, $P < 0.001$)

Predictor	B coefficient	B standard error	β	*t*-test	Significance
Serum phosphate (mg/dl)	49.5	18.3	0.4	2.6	0.02
PTHi/OPG	2.6	0.9	0.4	2.7	0.01
Age (years)	−12.9	4.2	−0.5	−3	0.01
Use of vitamin D (yes/no)	−245.7	99.5	−0.4	−2.4	0.03

Table 4. Differences in diverse variables when the patients were grouped according to serum bicarbonate (≤20 mEq/l *vs* >20 mEq/l) and vitamin D treatment

	Age (years)	Serum phosphate (mg/dl)	Phosphate removal (mg)	OPG (pmol/l)	RANKL/OPG	PTH/OPG
Low ($n=13$) *vs*	55.3 (10.6)	7.9 (2.6)	1302 (254.4)	14 (15.4)	0.1 (0.1)	65.9 (73.1)
high bicarbonate ($n=15$)	70.9 (11.2)	4.9 (0.7)	901.6 (119.7)	20.8 (11.5)	0.05 (0.06)	20.2 (17.3)
P	0.009	0.000	0.000	0.3	0.01	0.07
PTH/OPG < 10.7 ($n=9$) *vs*	66.5 (9.4)	5.7 (1.8)	1058.1 (228.7)	20 (14.1)	0.08 (0.08)	6.5 (3)
PTH/OPG > 40 ($n=9$)	59.2 (14.3)	8 (3)	1303.3 (354.2)	8.7 (4.3)	0.1 (0.1)	97.4 (47.1)
P	0.2	0.1	0.1	0.03	0.3	0.001
Yes ($n=9$) *vs* No vitamin D ($n=19$)	74.4 (6.4)	5.1 (1)	1049 (283.4)	22.2 (13.1)	0.04 (0.04)	30.3 (33.7)
	60 (13.1)	6.3 (2.4)	1107.9 (295.6)	13.2 (11.3)	0.1 (0.1)	47.4 (55.1)
P	0.005	0.2	0.6	0.07	0.05	0.4

OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor (NF)-κB ligand.
To convert serum phosphate from mg/dl to mmol/l, multiply by 0.32.

Discussion

The present study provides a set of new data aimed at analysing the relationship between several markers of bone activity and dialysis of phosphate. A main outcome of the study was the finding of an association of some of these markers with phosphate removal by haemodialysis. However, a critical fact was that the role of these molecules is subordinated to that of the main determinant of phosphate elimination, namely, plasma phosphate concentration.

To the best of our knowledge, the present study is the first to examine the potential role of bone activity and related molecules on dialytic phosphate clearance. A second outcome of the study was that none of the markers analysed appears to individually define the conditions of phosphate elimination. In fact, bone activity and phosphate removal are more efficiently described by a combination of factors. This finding suggests that there is an interaction, either stimulatory or inhibitory, which is actually relevant to determine the actual phosphorus elimination.

The available reports on phosphate kinetics in HD, still do not fully explain the basis of phosphate turnover. In our study, positive predictive factors of phosphate removal were serum phosphate and PTHi/OPG ratio, albeit in unequal proportions. As mentioned, serum phosphate has been found to be the critical factor in determining phosphate removal. In this regard, the relationship between pre-HD serum phosphate and the total amount of phosphate dialysed is readily understandable and our results fully confirm this point. Accordingly, the finding of higher phosphate removal in groups with higher serum phosphate, e.g. younger people or individuals with lower bicarbonate or lower OPG, was the predictable outcome. Another example of interest is the absence of significant differences in phosphate removal between the groups with extreme patterns of high and low bone turnover. The reason that upholds this finding, which apparently denies the importance of bone turnover in phosphate elimination, with the role of PTHi/OPG, which supports the significance of bone activity-related factors, could be the much higher relative weight of pre-dialysis plasma phosphate in determining phosphate removal (see also next paragraph). Moreover, the small number of patients in each high and low turnover group precludes a more definitive conclusion on this particular aspect.

Nonetheless, multiple regression analysis indicates that factors other than serum phosphate have also influence, and should be taken into account in depicting the full scenario of phosphate turnover regulation. The ratio PTHi/OPG was used as representative of the balance between bone resorption and formation stimuli. In our mathematical model, the PTHi/OPG quotient was able to explain phosphate elimination independently; for this reason, additional attention was focused on this ratio (Table 4). The results obtained indicate that PTHi/OPG ratio is positively related

with phosphate removal, bAP and TRACP-5b. In this ratio, PTHi is representative of bone reabsorption, which could be putatively counter-balanced by the anti-resorptive effects of OPG, leading to patterns of mineral mobilization that cannot be appraised by the more traditional markers. However, a full interpretation of the precise role of PTHi/OPG ratio in phosphate turnover is beyond the scope of the present study, and a caveat should be raised on the actual significance of the circulating levels of these and some other molecules, e.g. RANKL. The alternative, that PTHi and OPG affect more labile pools of phosphate, e.g. extra osseous, should also be taken into consideration.

According to our own and other's results, HD patients have higher OPG levels than their healthy controls. Also, the positive relationship between OPG and age found in normal individuals is maintained in end-stage renal disease [12]. At present, the mechanisms that regulate OPG secretion are largely unknown.

However, the precise role of the elevated OPG in dialysis patients is still unclear. In light of our results, it can be speculated that OPG increases as a compensatory mechanism, in an attempt to compensate the enhanced resorptive activity in secondary hyperparathyroidism; alternatively, since its levels were higher in the vitamin D-treated group, OPG may have increased as a response to vitamin D treatment. However, the fact that vitamin D-treated patients were significantly older casts a reasonable doubt on the reliability of attributing the increased OPG to vitamin D.

With regards to RANKL, to the best of our knowledge, the present one is the first report on plasma levels of RANKL in HD patients. Reference values from healthy volunteers were between 0 and 2.7 pmol/l, and were similar in transplant patients [17]. These values are in the range of what we have found in HD patients. Although, no relationship was detected between serum RANKL and parameters of bone activity, our results, as previously explained regarding OPG, suggest that RANKL/OPG ratio may be more relevant than the values of each variable considered separately. Nonetheless, the utility of this measurement in the HD setting remains undefined; *a priori*, the absence of relationship between serum RANKL and bone activity markers could indicate that RANKL alone is not a good marker of bone turnover.

The finding that the RANKL/OPG ratio was higher in patients with lower bicarbonate can be related to recent data describing a $[H^+]$-sensing receptor in bone cells. Acidosis has been shown to increase early immediate response genes in osteoblasts and to increase the receptor activator of NF-κB ligand expression and osteoclastic bone resorption [18]. This feature is on the line of the higher values of RANKL related to lower bicarbonate, without changes in OPG, found in the present study. Further studies are necessary to examine whether this finding is meaningful for the controversial issue of the role of acid-base equilibrium in regulating bone turnover in ESRD. Extracellular acidosis promotes the egress of phosphate from cells, inducing a slight rise in serum phosphate concentration [19]. Accordingly, the dialysate-to-plasma concentration gradient augments and favours phosphate removal; accordingly, acid-base changes impact on the ability of dialysis to maintain phosphate homoeostasis. Therefore, it is still not possible to firmly establish whether acidosis promotes higher phosphate concentrations only by its actions on bone cells or by effects on several cellular pools.

Some limitations of this study should be mentioned. First among them are the lack of bone biopsies and the relatively small number of patients. However, the use of validated indirect biochemical methods to assess bone formation or resorption may efficiently substitute for the bone histology [7]. Second, a longitudinal design will be required to give a more definitive description of the relevant relationships between bone turnover and phosphate dialysis. Furthermore, and as previously mentioned, the lack of completely reliable methods to assess phosphate pools located in different tissues, precludes a more definitive answer to the issue of the actual sources of the dialysed phosphate.

In conclusion, our data emphasize the complexity of the process regulating phosphate removal by HD. Even though initial serum phosphate is the principal among the involved factors, other, such as PTH/OPG, have a relevant contributory role. Furthermore, the finding of higher RANKL/OPG ratio in patients with lower bicarbonate points to the implication of acid-base changes in bone turnover by mediation of RANKL stimulation. Finally, serum RANKL levels are reported in HD patients and are in the range of normal individuals.

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Conflict of interest statement. None declared.

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