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Association between Biomarkers of Mineral and Bone Metabolism and Removal of Calcium and Phosphate in Hemodialysis

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Keywords
Bone turnover markers · Hemodialysis · Osteocalcin · Parathyroid hormone · Phosphate and calcium kinetics

Abstract
Background: A significant drop of serum phosphate and calcium removal or loading during hemodialysis induce reactions in mineral and bone remodeling that may inversely affect phosphate and calcium removal during dialysis. ***Objectives:*** We aimed to analyze the interdependencies between biomarkers of mineral and bone metabolism and removal of phosphate and calcium during hemodialysis, as this complex relationship is not fully understood. ***Methods:*** Three subsequent hemodialysis sessions during a 1-week treatment cycle with interdialytic periods of 2–2-3 days were monitored in 25 anuric patients. Calcium and phosphate concentrations were measured in serum before, at 1, 2, and 3 h, at the end, and 45 min after each session and in the outlet dialysate every 30 min. Biomarkers associated with mineral and bone metabolism: parathyroid hormone (PTH 1–34 and PTH 1–84), calcitonin, 25(OH)-vitamin D, fetuin-A, osteopontin, osteocalcin 1–43/49, and intact osteocalcin were assayed once in each patient before the midweek hemodialysis session. ***Results:*** Post-dialytic and intra-dialytic serum phosphate of midweek hemodialysis session and phosphate mass removed within 1 week correlated positively with serum PTH ($0.40 < \text{rho} < 0.46$, p value < 0.05). Higher concentration of serum PTH was associated with an increased level of osteocalcin. Pre-dialytic, post-dialytic, average for treatment time and average weekly concentrations of ionized calcium in serum correlated positively with serum osteocalcin. Serum osteocalcin and osteopontin levels were associated with the masses of total and ionized calcium, respectively, removed during 3 hemodialysis sessions. ***Conclusions:*** During hemodialysis, phosphate removal was associated with serum PTH, whereas calcium kinetics was influenced by serum osteocalcin and osteopontin. These results demonstrate that active processes involving biomarkers of mineral and bone metabolism are affected by the phosphate and calcium kinetics already within 4 h hemodialysis sessions.

Introduction

Achieving acceptable phosphate and calcium mass balance, which is crucial for controlling chronic kidney disease – mineral and bone disorders (CKD-MBD) in patients undergoing hemodialysis treatment, still poses great chal-

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lenges. One problem is that the efficiency of phosphate removal by hemodialysis performed 3 times per week is reported to be insufficient to balance the dietary phosphate intake [1–4]. Efforts are therefore made to control the dietary intake of phosphate (especially to restrict phosphate-containing food additives) and additionally, the majority of dialysis patients take oral phosphate binders to lower the load of phosphate [5, 6]. While calcium content in dialysis fluid can be tailored to prevent too high or too low serum calcium concentration [1, 7–9], the effects on calcium mass balance often remain unclear. Studying phosphate and calcium kinetics during hemodialysis may give better insights into the overall mass balance of phosphate and calcium; however, the resulting mass balance is influenced by several active processes governed by many control mechanisms.

CKD-MBD has a major role in the high mortality of CKD patients and when analyzing mortality risk factors, it is important to consider many parts of the complex system of parameters involved in CKD-MBD rather than single features such as phosphate [5, 10–13]. In patients with end-stage renal disease, phosphate loading and deficiency of vitamin D may increase serum parathyroid hormone (PTH), which, in turn, causes an increase in the circulating concentration of fibroblast growth factor-23, FGF-23 [14]. The whole system of interactions between organs, controlled by different regulatory and feedback mechanisms, is very complex and has implications for clinical outcomes [14–16].

Many studies have shown that, in the long term, calcium and phosphate levels affect PTH secretion and consequently bone turnover; however, there is a limited knowledge about the interactions of calcium and phosphate with other biomarkers associated with mineral and bone turnover during hemodialysis [13, 15, 16]. We hypothesized that differences in phosphate and calcium kinetics in hemodialysis patients may be due to differences in circulating factors that regulate tissue mobilization, intestinal absorption and bone remodeling. In the present study, we analyzed the interdependencies between parameters describing calcium and phosphate kinetics during a weekly cycle of 3 hemodialysis sessions and biomarkers connected with bone and mineral metabolism, including PTH, calcitonin, vitamin D, fetuin-A, osteopontin, and osteocalcin.

Materials and Methods

Patients and Biomarkers Related to Mineral and Bone Metabolism

Three consecutive hemodialysis sessions, with interdialytic breaks of 2–2-3 days, were monitored in 25 anuric patients (Table 1, Fig. 1). All patients underwent their regular treatment with an average ultrafiltration rate of 10.6 ± 3.5 mL/min. Blood flow range was 200–360 mL/min and dialysate flow 500 mL/min. The session time was set to 4 h and was on average 239 ± 11 min. The volume of processed blood was calculated as blood flow multiplied by treatment time (Table 1). All patients had arteriovenous fistulas. The studied group of patients was previously described in terms of calcium [17] and phosphate kinetics [18–20].

Table 1. Basic characteristics of the studied group of patients. Weight, body mass index and biochemical measurements in serum reported for the end of midweek hemodialysis session (median and 10th–90th percentiles)

Patients (n = 25)	Median (10th–90th percentiles)
Gender, male, %	36
Age, years	66 (43.4–79.6)
Time on dialysis, year	12 (2.0–25.6)
Height, cm	167 (154.4–175.6)
Weight, kg	65.7 (47.7–89.2)
Body mass index, kg/m^2	23.8 (19.7–30.6)
Processed blood volume, L/session	67.2 (48.0–83.0)
Biochemical measurements in serum	
Potassium, mmol/L	4.2 (3.9–4.7)
Sodium, mmol/L	141.0 (137.4–143.0)
Magnesium, mmol/L	0.86 (0.83–0.97)
Albumin, mmol/L	0.63 (0.54–0.71)
Creatinine, mmol/L	0.27 (0.21–0.38)
Urea, mmol/L	6.09 (4.53–8.18)
Urea spKt/V	1.5 (1.3–1.6)

Inorganic phosphorus (by Advia 1800, Siemens), and total (by calorimetric method with Arsenazo, Advia 1800, Siemens) and ionized (by ion selective electrode and potentiometry direct mode, RapidLab 348, Siemens) calcium concentrations were measured in serum before, at 1, 2, and 3 h, at the end, and 45 min after each session, before the fourth hemodialysis session, and in the dialysate outlet every 0.5 h. The nominal concentration of calcium in bicarbonate buffered dialysis fluid was 1.25 mmol/L. In all patients, acetic acid was used as acidifier of dialysis fluid. Sevelamer hydrochloride was prescribed in 7 (28%), aluminum hydroxide in 7 (28%) of patients and all patients received calcium carbonate with average daily intake of 3.1 ± 1.9 g. Cinacalcet was applied in 7 patients (30 mg/day in each) and 10 patients (40%) received alphacalcidolum with average daily dose of 0.6 ± 0.3 μg.

Biomarkers associated with mineral and bone metabolism were assayed in each patient before the midweek session. The list below contains the measured biomarkers and the measurement method:

(a) PTH 1–34 (enzyme-linked immuno-sorbent assay, ELISA Kit, Immutopics, Inc., San Clemente, CA, USA),

(b) PTH 1–84 (ELISA Kit, Immutopics, Inc., San Clemente, CA, USA),

(c) Calcitonin (bone health enzyme immunoassay, EIA Kit, Quidel Corporation, San Diego, CA, USA),

Table 2. Biomarker concentrations in blood serum (mean ± SD, range and normal range) and their reciprocal correlations (Spearman rho and *p* value)

	Biomarker	Mean ± SD	Rho[p value]	Range	Normal range[#]
(a)	PTH 1-34, pg/mL	24.3±35.6	(b) 0.96***, (h) 0.52**	0.00–113.6	0–165
(b)	PTH 1-84, pg/mL	295.5±379.5	(a) 0.96***, (h) 0.56**	0.5–1,200.0	150–300
(c)	Calcitonin, pg/mL				
	Males	9.5±6.6		0–23.4	<13.8
	Females	2.1±4.3		0–16.9	<6.4
(d)	25(OH)-vitamin D, nmol/L	41.7±25.4		11.8–106.6	12–230
(e)	Fetuin-A, g/L	0.90±0.24	(f) –0.50*	0.59–1.46	0.33–1.0
(f)	Osteopontin, ng/mL	231.1±243.4	(e) –0.50*	12.9–813.8	5–320
(g)	Osteocalcin 1–43/49, ng/mL	133.5±50.0		45.2–248.4	0–64
(h)	Intact osteocalcin, ng/mL	79.0±44.6	(a) 0.52**, (b) 0.56**	4.9–140.5	3.7–10

[#] Range for healthy subjects according to the specification of the measurement method.
In brackets are shown biomarkers (letters) that correlate with the current position. *p* value: *** <0.001, ** <0.01 and * <0.05.

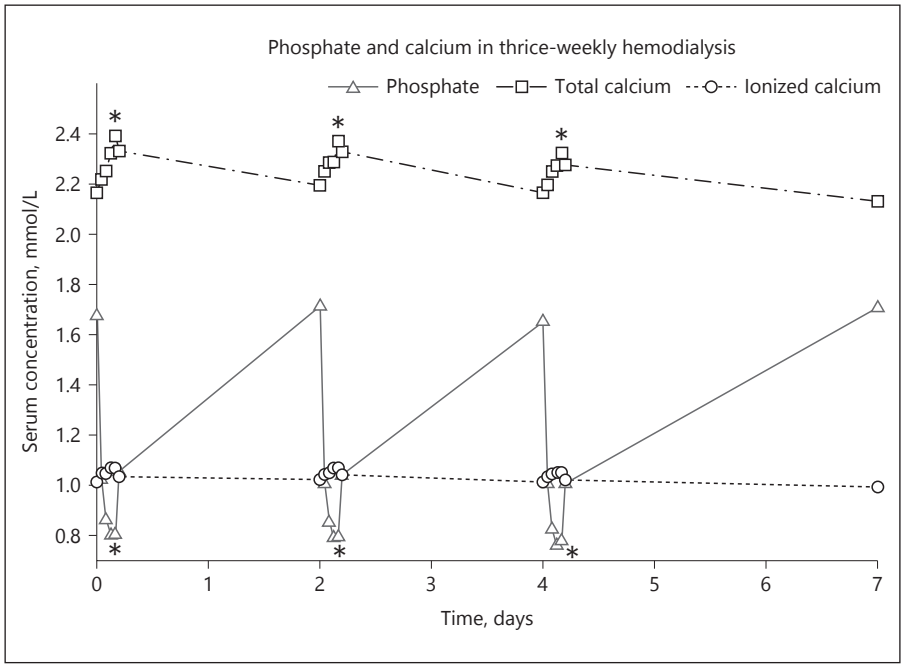

Fig. 1. Mean phosphate and calcium concentrations in serum during 1 week cycle of 3 consecutive hemodialysis sessions (*n* = 25 patients). * Significant change between post- vs. pre-dialytic concentration.

(d) 25(OH)-vitamin D (direct ELISA Kit, Immundiagnostik AG, Bensheim, Germany),

(e) Fetuin-A (ELISA Kit, Epitope Diagnostics, Inc., San Diego, CA, USA),

(f) Osteopontin (Immuno-Biological Laboratories Co., Ltd., Takasaki-Shi, Japan),

(g) Osteocalcin 1–43/49 (ELISA Kit, Epitope Diagnostics, Inc., San Diego, CA, USA),

(h) Osteocalcin intact (enzyme immunoassay, EIA Kit, Quidel Corporation, San Diego, CA, USA).

Each biomarker concentration in blood serum was measured in duplicates and the average was reported (Table 2).

Indices of Phosphate and Calcium Kinetics

Concentrations of phosphate and calcium were monitored during the whole weekly cycle of 3 hemodialysis sessions. For phosphate, total and ionized calcium: pre-dialytic ($C_{pre,midweek}$) and post-dialytic ($C_{post,midweek}$) concentrations, their ratio ($C_{post,midweek}/C_{pre,midweek}$) and rebound for midweek session (rebound = $C_{eq,midweek}/C_{post,midweek}$-1, where $C_{eq,midweek}$ is the concentration determined 45 min after the end of hemodialysis), treatment time averaged ($C_{mean,treatment}$, that is, average calculated for the dialysis duration) and time averaged concentration ($C_{mean,week}$, calculated for the whole week) are reported.

The removed mass (M_d) of calcium and phosphate for midweek session ($M_{d,midweek}$) and for the whole week ($M_{d,week}$) were

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Table 3. Associations between parameters of phosphate and calcium kinetics and biomarkers of mineral and bone metabolism

	Phosphate	Rho$^{p\ value}$	Total calcium	Rho$^{p\ value}$	Ionized calcium	Rho$^{p\ value}$
$C_{pre,midweek}$, mmol/L	1.71±0.57		2.2±0.19	(h) 0.46*	1.02±0.14	(h) 0.60**
$C_{post,midweek}$, mmol/L	0.80±0.21	(a) 0.44*, (b) 0.46*	2.37±0.2	(c) 0.47*	1.07±0.12	(h) 0.52**
$C_{post,midweek}/C_{pre,midweek}$	0.49±0.11		1.08±0.1	(c) 0.40*, (f) –0.43*	1.05±0.11	(f) –0.52**
$Rebound_{midweek}$	0.30±0.07		–0.02±0.07	(f) 0.40*	–0.03±0.05	(c) –0.48*, (f) 0.43*
$C_{mean,treatment}$, mmol/L	0.97±0.28	(b) 0.43*	2.26±0.13		1.05±0.11	(h) 0.60**
$C_{mean,week}$, mmol/L	1.33±0.41		2.24±0.13	(h) 0.44*	1.02±0.12	(h) 0.61**
$M_{d,midweek}$, mmol/session	29.73±11.12	(f) –0.44*	1.10±8.19	(d) 0.49*	–5.49±4.64	(f) 0.45*
$M_{d,week}$, mmol/week	88.17±29.81	(a) 0.40*, (f) –0.42*	5.67±13.49	(h) 0.41*	–13.53±12.17	(f) 0.45*
ECC, mL/min	6.64±0.86	(e) 0.43*	0.25±0.6	(h) 0.40*	–1.38±1.29	(f) 0.43*, (h) 0.43*

For phosphate, total calcium and ionized calcium presented are: pre-dialytic ($C_{pre,midweek}$), post-dialytic ($C_{post,midweek}$), ratio of post-dialytic to pre-dialytic concentration ($C_{post,midweek}/C_{pre,midweek}$) and rebound for midweek session, average concentration calculated only for dialysis duration ($C_{mean,treatment}$) and average concentration for the whole week ($C_{mean,week}$), mass removed during midweek session ($M_{d,midweek}$) and during the whole week ($M_{d,week}$), ECC and their correlations (rho and *p* value) with biomarkers listed in Table 2: (a) PTH 1-34, (b) PTH 1-84, (c) calcitonin, (d) 25(OH)-vitamin D, (e) fetuin-A, (f) osteopontin, (g) osteocalcin 1-43/49 and (h) intact osteocalcin. Negative sign of the removed mass and in ECC means calcium absorption to the body from dialysis fluid. *p* value: ** <0.01 and * <0.05.

ECC, equivalent continuous clearance.

calculated from the concentrations at the inlet ($C_{d,in}$) and outlet ($C_{d,out}$) of dialyzer and the inlet (Q_d) and outlet ($Q_d + Q_{UF}$) flow rates of dialysis fluid:

$$M_d = \int_0^T (Q_d + Q_{UF}) C_{d,out}(t)dt - \int_0^T Q_d \cdot C_{d,in}(t)dt$$

where Q_{UF} is the ultrafiltration rate and T is the time of hemodialysis session. $M_{d,week}$ was calculated as the sum of masses removed by 3 consecutive hemodialysis treatments. For phosphate, $C_{d,in}$ in equation (1) is equal to zero, as there is no phosphate in fresh dialysis fluid.

Equivalent continuous clearance (ECC) [21–23] was determined as the rate of solute removal (i.e., removed mass over time, $M_{d,week}/t$) over the time averaged solute concentration ($C_{mean,week}$):

$$ECC = \frac{M_{d,week}/t}{C_{mean,week}}$$

For both solutes (phosphate and calcium), ECC was determined for a 1-week cycle (*t* = 1 week).

Statistical Analysis

Statistical dependence between 2 variables was analyzed by Spearman's correlation coefficient (rho). In a multivariate analysis, the stepwise linear regression method was applied with R-squared based criterion for predictor addition or removal (0.05 threshold for adding and 0.01 for removing variable) to examine dependencies between calcium and phosphate removal by hemodialysis and various combinations of other data. Statistical analyses were performed in MATLAB R2018b (MathWorks, Natick, MA, USA) with the "stepwiselm" function embedded within.

Results

Reciprocal relationships between biomarkers included a negative association between osteopontin and fetuin-A, and positive association between PTH (1–34 and 1–84) and intact osteocalcin (Table 2). PTH 1–34 correlated with PTH 1–84 as expected (Table 2). Age correlated negatively with fetuin-A (rho = –0.54, *p* value <0.01) and 25(OH)-vitamin D (rho = –0.45, *p* value <0.05).

Post-dialytic ($C_{post,midweek}$) and mean treatment ($C_{mean,treatment}$) serum concentrations, and also the removed mass ($M_{d,week}$) of phosphate, correlated positively with the PTH level in serum (Table 3). The mass of phosphate removed during the midweek hemodialysis session, and during the whole week, was negatively associated with serum osteopontin (Table 3). ECC of phosphate correlated positively with serum fetuin-A.

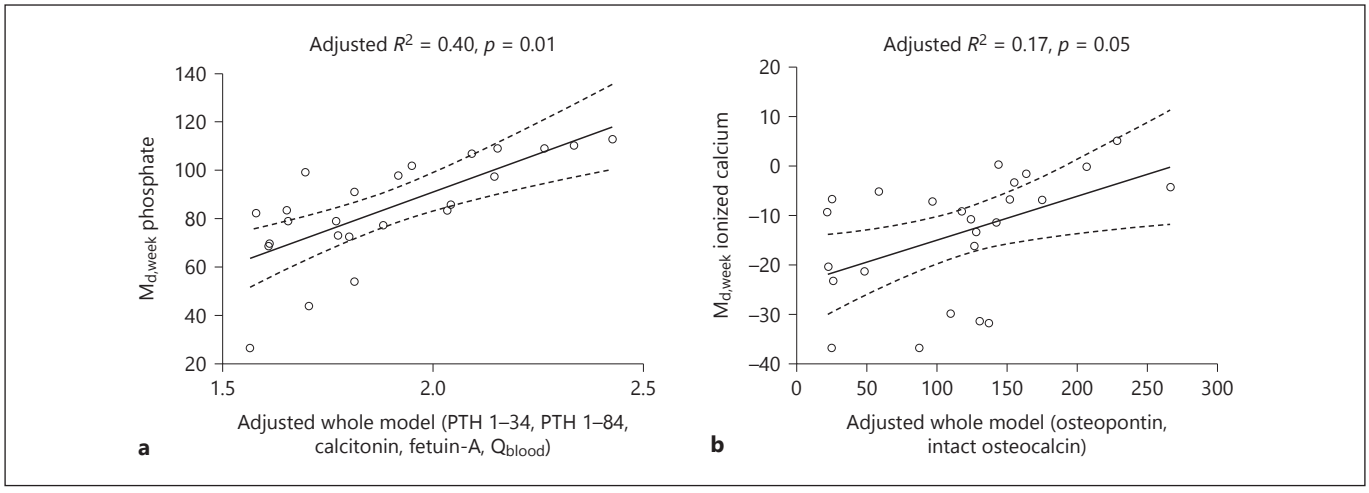

Fig. 2. The fit of multivariate models that predict weekly removal ($M_{d,week}$) of phosphate (**a**) and ionized calcium (**b**). Presented is regression line (solid line) and 95% CI (dotted line) for the adjusted dependent variable versus the adjusted combination of variables that were selected as the best predictors using the stepwise procedure. Q_{blood}, the volume of processed blood; PTH, parathyroid hormone.

Pre-dialytic and average (weekly) serum concentration as well as removed mass and ECC of total calcium were associated positively with the level of intact osteocalcin (Table 3). The post-dialytic, and the ratio of post-dialytic to pre-dialytic concentration of total calcium correlated positively with serum calcitonin (Table 3). Serum 25(OH)-vitamin D correlated with the mass of total calcium removed during the midweek hemodialysis session (Table 3). Rebound of total and ionized calcium correlated positively, whereas the ratio of post-dialytic to pre-dialytic concentration correlated negatively, with the concentration of osteopontin (Table 3). During the midweek session, and in the whole week, ionized calcium ($M_{d,midweek}$ and $M_{d,week}$) was absorbed from dialysis fluid to the body and this absorption as well as ECC was positively associated with the serum osteopontin (Table 3). Pre-dialytic, post-dialytic, treatment time average, weekly average concentrations and ECC of ionized calcium correlated positively with the serum concentration of intact osteocalcin (Table 3).

In a multivariate analysis, we searched for predictors influencing the removal of phosphate and ionized calcium ($M_{d,week}$) during the thrice weekly hemodialysis treatment. Among 8 biomarkers listed in Table 2, biomarkers PTH 1–84, PTH 1–34, calcitonin and fetuin-A were found together with the volume of processed blood to be associated with the removed mass of phosphate (adjusted R-squared = 0.40, p value = 0.01, Fig. 2a). The absorption of ionized calcium from dialysis fluid to the body was dependent on the level of osteopontin and intact osteocalcin (adjusted R-squared = 0.17, p value = 0.05, Fig. 2b).

Discussion

Our study in patients undergoing hemodialysis revealed several interdependencies between parameters describing phosphate and calcium kinetics and selected biomarkers with known impact on mineral and bone metabolism (PTH, calcitonin, 25(OH)-vitamin D, fetuin-A, osteopontin and osteocalcin). These results suggest that the availability of these circulating compounds, which have important roles as regulators of mineral homeostasis and bone remodeling, may influence phosphate and calcium removal during hemodialysis.

Phosphate

In our study, the post-dialytic and intra-dialytic serum phosphate concentrations during midweek hemodialysis session were associated with the pre-dialytic level of PTH (Table 3). The average weekly removal of phosphate, $M_{d,week}$ = 88.17 ± 29.81 mmol, was comparable with previous reports on patients treated by standard hemodialysis [2, 24, 25] and in our study was positively correlated with PTH (Table 3). The positive association between phosphate and PTH is in agreement with previous observations that serum phosphate concentrations correlate

with the level of PTH, a hormone that increases phosphate release from bone [15, 26, 27]. Serum PTH, being a marker of bone turnover status, is closely associated with calcium and phosphate derangements and serves as one of the main targets – and a key marker of the effects – of pharmaceutical interventions aiming at reducing phosphate levels in patients with CKD [28]. Additionally, for hemodialysis, Albalate et al. [29] found that phosphate removal depended on the ratio of PTH-to-osteoprotegerin. In our study, the removed mass of phosphate correlated negatively with serum osteopontin – a marker of bone resorption and formation – perhaps implicating that an imbalanced bone remodeling with elevated serum osteopontin levels was associated with a decrease of dialytic removal of phosphate [30]. These relations are in stark contrast to the opposite (i.e., positive) correlations for osteopontin versus calcium removal, vide infra. In a multivariate analysis, the weekly removal of phosphate was dependent on serum PTH, calcitonin, fetuin-A and the volume of blood processed during the treatment with the last factor related to the influence of dialysis intensity on phosphate removal (Fig. 2a). Urea KT/V, which was in our study 1.48 ± 0.15, did not contribute to the prediction of phosphate removal, suggesting once more that dialysis adequacy assessed by urea KT/V is not a surrogate measure of phosphate removal. Our results (Table 3) support the view that PTH is a determinant of serum phosphate while calcitonin – counteracting the actions of PTH (and vitamin D) – acts primarily to reduce the concentrations of serum calcium. However, fetuin-A, being a powerful inhibitor of vascular calcification, is also known to be associated with phosphate levels, and this could have potential implications for the dialytic removal of phosphate [31].

Calcium

Our study and several previous studies demonstrate that calcium removal during hemodialysis varies widely with high inter-patient and inter-session variability [17, 32, 33]. These findings suggest that calcium removal during hemodialysis depends not only on the dialysate calcium concentration but also on other individual factors [17, 32, 33]. One such factor is vitamin D; its concentration correlated (rho = 0.49; $p < 0.05$) with the mass of calcium removed during the midweek session (Table 3), reflecting the role of vitamin D as a key regulator of serum calcium concentration [34]. In the study by Karohl et al. [32], a multivariate analysis showed that calcium removal in hemodialysis was dependent on PTH and osteocalcin. In our study, the midweek pre-dialytic and post-dialytic concentrations of ionized calcium, treatment time, and mean weekly concentrations of ionized calcium in serum were dependent on the serum intact osteocalcin concentration measured before the midweek session (Table 3). The ratios of post- to pre-dialytic concentrations of total and ionized calcium were associated with serum osteopontin (Table 3). The masses of total and ionized calcium removed during the week correlated positively with circulating concentrations of intact osteocalcin and osteopontin respectively (Table 3). Also, the ECC for calcium was connected with serum osteopontin and intact osteocalcin (Table 3). In multivariate analysis, we found that the mass of ionized calcium removed within a week depended on the concentrations of osteopontin and osteocalcin (Fig. 2b). The above dependencies may indicate a significant role of osteopontin and osteocalcin in the calcium kinetics. Since osteopontin and osteocalcin are important regulators of bone remodeling as well as of vascular calcification, or "vascular ossification" [30, 35], these observations underscore the close links of calcium kinetics during hemodialysis with bone and vascular status as part of a bone-vascular axis. Overall, the current findings corroborate the important roles of bone reabsorption and bone formation processes, the activity of which are reflected by osteopontin and osteocalcin, respectively, in mineral metabolism (calcium kinetics in particular).

Some limitations of our study should be considered when interpreting the results. We analyzed a relatively low number of biomarkers of bone and mineral metabolism; all were selected due to their known relevance for CKD-MBD. However, we did not measure FGF-23, osteoprotegerin and bone-specific alkaline phosphatase to name a few. Another potential drawback of our study is the single measurement of the analyzed biomarkers; however, there are no guidelines for particular measurement schedules that would guarantee capture of cause-and-effect relationships influencing bone and mineral metabolism. Some processes are quick and tightly regulated, as the control of serum calcium, whereas other processes take time, as remodeling of bone [36]. Other problems include the circadian and seasonal fluctuations of biomarkers for mineral and bone metabolism [15, 37]. Moreover, in patients on dialysis, the system of parameters involved in mineral metabolism is affected by the schedule and parameters of the performed dialysis [2, 38] (compare Fig. 1). For instance, in the study by Ferraresi et al. [38], the high level of calcium in the dialysis fluid (1.75 mmol/L) resulted in significant mid-dialysis reduction in PTH, increase of serum calcium, and decrease of serum phosphate.

The strengths of our study include the very detailed clinical protocol of the monitoring of phosphate and calcium (total and ionized) kinetics during 3 consecutive hemodialysis sessions in 1-week cycle in anuric patients. Frequent sampling in blood (every hour) and dialysate (every half hour) during dialysis allowed us to catch the weekly profile of serum phosphate and calcium and calculate with high precision the removed mass, the continuous equivalent clearance, and other parameters of relevance for phosphate and calcium removal.

In summary, the significant drop of serum phosphate and calcium load or removal during hemodialysis seem to interact with processes involved in mineral and bone remodeling. The observed correlations suggest a significant role of PTH in phosphate removal, while osteopontin and intact osteocalcin appear to have a major impact on calcium kinetics. These results indicate that phosphate and calcium removal in patients undergoing hemodialysis is determined by several active processes regulated by many control mechanisms, and many of the examined biomarkers seem to have bidirectional or multidirectional functions. While some mechanisms become active already within a 4-h hemodialysis session, the analysis of the impact of some other mechanisms would require long-term observation studies. Such studies should ideally include assessments also of other factors that influence CKD-MBD that interact with calcium and phosphate kinetics in hemodialysis patients, such as dietary intake of phosphate, calcium and vitamin D, bone status and vascular calcification.

Statement of Ethics

The research was conducted ethically in accordance with the Declaration of Helsinki. The study protocol has been approved by the Bioethical Committee at the Medical University of Lublin (Poland, resolution number: KE-0254/179/2011). Subjects have given their written informed consent.

Disclosure Statement

B.L. is affiliated with Baxter Healthcare. The other authors declare no conflicts of interest.

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Author Contributions

J.W., M.D., and W.Z.: conceptualization; M.D.: project administration; M.D., J.W., and W.Z.: methodology; A.W.-Z.: acquisition of clinical data; M.D. and J.P.: calculations and visualization; M.D., L.D., B.L., J.P., and J.W.: analysis and interpretation of results; M.D.: writing first draft; L.D., M.D., B.L., J.P., A.W.-Z., J.W., and W.Z.: writing – review and editing.

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