Nephrol Dial Transplant (2006) 21: 999–1005
doi:10.1093/ndt/gfi308
Advance Access publication 2 December 2005

Original Article

NDT
Nephrology Dialysis Transplantation

Association of mineral metabolism with an increase in cellular adhesion molecules: another link to cardiovascular risk in maintenance haemodialysis?

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Abstract

Background. Abnormal mineral metabolism is associated with increased cardiovascular morbidity and mortality. The exact pathogenesis linking mineral metabolism to cardiovascular risk is unknown. This study was undertaken to investigate the association between serum phosphate and/or $Ca \times PO_4$ product with serum levels of soluble E-selectin (sE-selectin), soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 and the degree of carotid artery atherosclerosis in patients on haemodialysis.

Methods. Seventy-three patients (46 male, 27 female; mean age 48 ± 13 years, on haemodialysis for 82 ± 80 months) were included in the study. All patients were stable, had no evidence of vascular disease and/or active infection. Consecutive 6 months clinical and laboratory data were obtained for each patient from their medical records and mean values were used for analysis. Serum levels of soluble adhesion molecules were assayed by ELISA. All subjects underwent a detailed evaluation of the carotid arteries.

Results. The percentage of patients who met all three targets of NKF-K/DOQI for phosphate, calcium and $Ca \times PO_4$ product was 27.1%, whereas those who did not achieve the target in one, two or three parameters was 28.1, 17.7 and 14.6%, respectively. The sICAM-1 levels were significantly higher in patients who had hyperphosphataemia (serum phosphate >5.5 mg/dl; $P = 0.044$) and hypercalcaemia (serum calcium >9.5 mg/dl; $P = 0.014$), both sE-selectin and sICAM-1 levels were significantly higher in patients with $Ca \times PO_4$ product levels above $55\,mg^2/dl^2$ ($P = 0.002$ and $P = 0.000$, respectively). Soluble E-selectin and sICAM levels demonstrated a near-linear increase in parallel to the degree of deviation from mineral metabolism targets. Soluble E-selectin and sICAM levels were correlated with serum phosphate and $Ca \times PO_4$ product, but there were no correlations between adhesion molecules and carotid measurements.

Conclusion. These findings suggest that in stable haemodialysis patients abnormal bone mineral metabolism was associated with increased soluble adhesion molecules. These alterations in adhesion molecules may favour the development of cardiovascular changes and contribute to high cardiovascular morbidity and mortality in patients with abnormal mineral metabolism.

Keywords: adhesion molecules; atherosclerosis; calcium × phosphate product; carotid artery intima-media thickness; haemodialysis; mineral metabolism

Introduction

Elevated serum phosphate is a predictable consequence of renal failure and is present in most patients receiving dialysis [1]. Despite substantial improvements in dialysis applications, hyperphosphataemia is still a very prevalent problem with 70% of patients having serum phosphate above normal ranges (2.5–4.5 mg/dl), and 30% having values higher than 7.0 mg/dl [2]. Hyperphosphataemia causes many deleterious effects in dialysis patients and classically most of them are attributed to the development of renal osteodystrophy. Block *et al.* were the first to report elevated serum phosphate values greater than 6.5 mg/dl as an independent predictor of mortality among people who receive

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chronic dialysis [2]. A follow-up data of the same cohort showed that hyperphosphataemia, as well as an elevated $Ca \times PO_4$ product was particularly associated with deaths resulting from coronary artery disease [3]. Hyperphosphataemia and elevated $Ca \times PO_4$ product are, thus, thought to represent novel cardiovascular risk factors in uraemic patients [4].

The exact mechanisms whereby hyperphosphataemia and/or elevated $Ca \times PO_4$ product contributes to increased cardiovascular risk remain to be determined; the most frequently quoted mechanism is widespread cardiovascular calcification [5]. Several *in vitro* and *in vivo* studies demonstrated that serum phosphate and calcium levels, as well as inflammation, imbalance between calcification promoters and inhibitors and uraemic state *per se*, are actively involved in the calcification process [6]. Vascular calcification in end stage renal disease (ESRD) is associated with vascular stiffening, ischaemic heart disease and increased atherosclerosis, thus, a poor outcome [7–8].

Atherosclerosis is known to be an inflammatory disorder and it has an accelerated course in ESRD [9]. The mechanism by which uraemia accelerates the atherosclerotic process is not well understood, but altered pro- and anti-inflammatory cytokines and endothelial dysfunction have an important role. Cell adhesion molecules (CAM), which are expressed on the surface of vascular endothelium in response to pro-inflammatory cytokines and mediate blood cell (leukocyte, platelets)–endothelial cell interactions, have been implicated in the pathogenesis of atherosclerosis [10]. Soluble forms of adhesion molecules have been detected in serum and reported to be indicative of the expression of membrane bound adhesion molecules [11]. It was suggested that endothelial activation and inflammation occurs early in the atherosclerotic process and that high serum levels of adhesion molecules may predict future cardiovascular events [12,13].

The purpose of this study was to seek out the relationship between serum phosphate and/or $Ca \times PO_4$ product and serum levels of CAM, namely soluble E-selectin (sE-selectin), soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) and the degree of carotid artery atherosclerosis in stable ESRD patients on haemodialysis.

Materials and methods

Patients

The study included 73 stable, non-diabetic, ESRD patients (46 male, 27 female; mean age 48 ± 13 years) on haemodialysis for at least 12 months (mean $\pm$ SD: 82 ± 80 months, range: 12–296 months). Patients were selected from a total population of 134 patients treated at our centre. In order to eliminate major confounding factors of atherosclerosis and/or inflammation, patients with a history of acute myocardial infarction (MI), valvular heart disease, heart failure, cerebral or peripheral vascular disease and common carotid artery stenosis or active infection/inflammation were excluded.

All patients were dialysed for 4 h, three times/week, using hollow-fibre synthetic membranes (Polysulphone F6 and F7, Fresenius) with a bicarbonate dialysate containing 1.25 mmol/l calcium. Fifty-eight patients were being regularly treated with recombinant human erythropoietin and 17 were on antihypertensive therapy. Patients were regularly taking iron and vitamin supplements. All patients were on phosphate binders: 36 were on calcium carbonate (4.6 ± 1.9 g/day), 33 were on calcium acetate (3.1 ± 1.1 g/day), two were on aluminium hydroxide (3.7 ± 1.1 g/day) and 2 were on sevelamer (2.0 ± 0.6 g/day). Twenty-one patients were on 1,25-vitamin D3 with a mean dose of 0.39 ± 0.11 μg/day. The study was carried out in accordance with the Declaration of Helsinki and informed consent was obtained from all patients.

Laboratory methods

Consecutive 6 months clinical and laboratory data were obtained for each patient from their medical records and the mean values were used for analysis. In the unit, a multiple-test laboratory panel (including blood urea nitrogen, creatinine, electrolytes, calcium, phosphate, liver enzymes, albumin and lipid profile) and complete blood count were evaluated by obtaining arteriovenous fistula blood in the first week of each month before a mid-week dialysis session. The intact parathyroid hormone (iPTH), C-reactive protein (CRP) and iron status (ferritin and transferrin saturation) were assessed every 2 months by standard methods.

Blood pressure data were collected from consecutive 6 month predialysis measurements. Blood pressure was measured with a mercury sphygmomanometer with standard techniques. Mean systolic, diastolic, arterial pressure and pulse pressures were calculated and used for further analysis.

Serum levels of soluble adhesion molecules were assayed in pre-dialysis arteriovenous blood corresponding with the 6 month blood sampling for clinical parameters. Serum was withdrawn following centrifugation at $+4^{\circ}C$ for 10 min at 2500 rpm and was stored at $-20^{\circ}C$ until assayed. Serum concentrations for sE-selectin, sICAM-1 and sVCAM-1 were determined by enzyme-linked immunosorbent assay (ELISA) using standard kits (human sE-selectin ELISA, human sICAM-1 ELISA and human sVCAM-1 ELISA, Bender MedSystems, Vienna, Austria). Sera were diluted 1:5, 1:50 and 1:100, respectively, for the quantization of sE-selectin, sICAM-1 and sVCAM-1. The serum concentrations of these soluble adhesion molecules were calculated by reference to standard curves obtained with the corresponding recombinant molecules. For all assays, the intra- and inter-assay coefficients of variation were less than 5 and 10%, respectively. All results from ELISA assays represent means from duplicated measurements and were expressed in nanograms per millilitre.

Carotid imaging

Each patient underwent a detailed ultrasound evaluation of the carotid arteries. These examinations were performed by an experienced radiologist using equipment generating a wide band ultrasonic pulse with a middle frequency of 7.5 MHz (Toshiba SSA-270 A, Tokyo, Japan). Carotid images were obtained with the patients in the supine position with the neck mildly extended and the head rotated contralaterally to the side. The imaging protocol involved obtaining longitudinal,

lateral and anterior oblique views of the distal 10 mm of the right and left common carotid arteries, the carotid bifurcation and the internal carotid artery. A mean intima-medial thickness (IMT) was computed in each region and for the purpose of statistical analysis, right and left measurements were averaged. The mean maximum IMT of the carotid bifurcations and the common carotid arteries, CBM(max), and the presence of well-defined atherosclerotic plaques were used as main measures to define carotid artery atherosclerosis. Plaques were defined by the presence of focal, severe wall thickening (IMT >2.0 mm), protrusion into the vascular lumen more than 1.5 mm, and calcification. In each investigation, the common carotid artery wall-to-lumen ratio was also calculated from both sides and mean values were used for analysis. The ultrasound operator was unaware of the clinical and laboratory data of the patients. All examinations were done during the 6th month of the study. Intraobserver reproducibility of the IMT measurements was evaluated in a subset of patients ($n = 15$).

Statistical analysis

The data were expressed as mean ± SD. All data were first analysed for normality of distribution using the Kolmogorov–Smirnov test for normality. Student's *t*-test was used to compare two groups and one-way ANOVA for multiple groups. Pearson's correlation coefficient (r) was calculated and tested for significance of linear relationship among variables. A multivariate regression analysis was carried out to assess the independent contribution of soluble adhesion molecules and mineral metabolism parameters on carotid atherosclerosis parameters. A P value <0.05 was considered to be significant. All data were analysed using SPSS V 10.0 for Windows (SPSS Inc).

Results

In the whole group, mean serum phosphate level was 4.97 ± 0.87 mg/dl, mean serum calcium level was 9.60 ± 0.80 mg/dl and $Ca \times PO_4$ product was 47.8 ± 9.1 mg^2/dl^2. The percentage of patients above National Kidney Foundation – Kidney Disease Outcomes Quality Initiative targets [14] for these parameters was 31.8% for phosphate (>5.5 mg/dl), 54.8% for calcium (>9.5 mg/dl) and 17.9% for $Ca \times PO_4$ product (>55 mg^2/dl^2). The percentage of patients who met all three targets was 27.1%, whereas in those who did not achieve the target in one, two or three parameters it was 28.1, 17.7 and 14.6%, respectively.

Serum adhesion molecules, namely sE-selectin, sICAM-1 and sVCAM-1 levels were higher in patients who were above NKF-K/DOQI targets for phosphate, calcium and $Ca \times PO_4$ product. However, the differences were only significant for sICAM-1 in all groups and for sICAM-1 and sE-selectin in patients with $Ca \times PO_4$ product levels above 55 mg^2/dl^2. The sICAM-1 levels were significantly higher in patients who had hyperphosphataemia (serum phosphate >5.5 mg/dl; $P = 0.044$); and hypercalcaemia (serum calcium >9.5 mg/dl; $P = 0.014$), both sE-selectin and sICAM-1 levels were significantly higher in patients

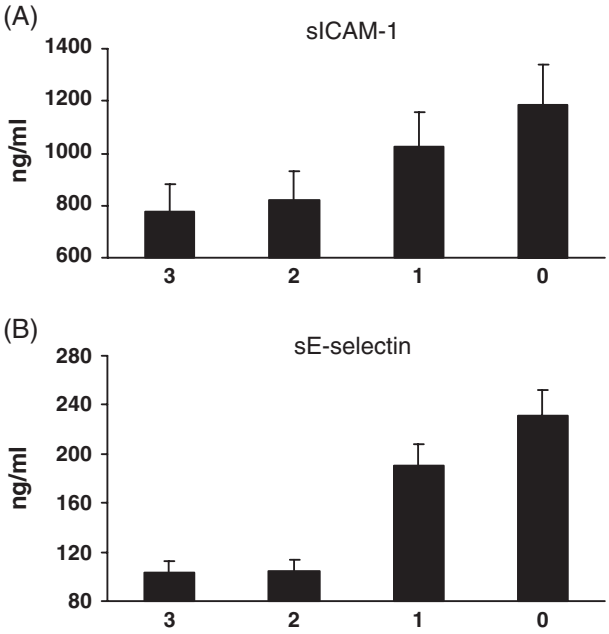

Fig. 1. Serum concentrations (mean ± SD) of (A) sICAM-1 and (B) sE-selectin according to number of mineral metabolism in target (3: all three in target, 2: two parameters in target, 1: only one parameter in target and 0: none in target) ($P = 0.002$ for sICAM-1 and $P = 0.021$ for sE-selectin).

with $Ca \times PO_4$ product levels above 55 mg^2/dl^2 ($P = 0.002$ and $P = 0.000$, respectively). When patients were grouped according to their level of achieving targets (0 to all 3 in target), sE-selectin and sICAM-1 levels demonstrated a near-linear and significant increase ($P = 0.021$ and 0.002, respectively) in parallel to the degree of deviation from these targets (Figure 1).

Patients were then separated into two groups according to NKF-K/DOQI targets for $Ca \times PO_4$ product: those with >55.0 mg^2/dl^2 and with $Ca \times PO_4$ product ≤55.0 mg^2/dl^2. The two groups were comparable for all demographic and clinical parameters, except higher predialysis serum calcium (10.1 ± 0.7 *vs* 9.3 ± 0.7 mg/dl, $P < 0.001$) and higher phosphate (5.7 ± 0.6 *vs* 4.6 ± 0.7 mg/dl, $P < 0.001$) levels in patients with elevated $Ca \times PO_4$ product (56.8 ± 4.3 *vs* 42.5 ± 5.6 mg^2/dl^2, $P < 0.001$). Both groups have similar dialysis adequacy measures (Kt/V was 1.28 ± 0.1 *vs* 1.24 ± 0.1 pg/ml, $P = 0.8$) and blood pressure control (mean arterial pressure was 91 ± 11 *vs* 93 ± 14 mmHg, $P = 0.6$). Intact PTH levels were higher in patients with $Ca \times PO_4$ product ≤52.0 mg^2/dl^2, but the difference was not significant (297 ± 345 *vs* 158 ± 241, $P = 0.056$). Serum albumin and CRP levels were not different between the two groups.

Mean IMT values were similar in patients with normal and elevated $Ca \times PO_4$ products (0.65 ± 0.03 mm and 0.66 ± 0.03 mm, respectively, $P > 0.05$). There was no difference in the mean maximum IMT of the carotid bifurcations, CBM (max) and the presence of atherosclerotic plaques between the two groups. However, mean IMT and

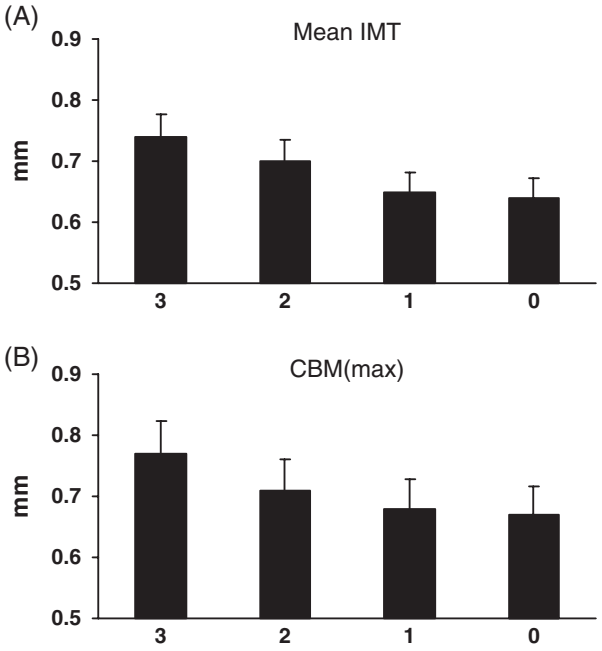

Fig. 2. Mean IMT and (B) mean maximum IMT of the carotid bifurcations and the common carotid arteries, CBM(max), according to number of mineral metabolism in target (3: all three in target, 2: two parameters in target, 1: only one parameter in target and 0: none in target) ($P = 0.04$ for IMT and $P = 0.04$ for CBM(max)).

CBM(max) levels demonstrated a significant increase when all three mineral metabolism parameters were not in NKF-K/DOQI targets (Figure 2). There were 19 patients with a carotid plaque. Plaque positive patients were older (56 ± 8 *vs* 41 ± 9, $P < 0.05$), had significantly higher IMT and CBM(max) measurements (0.77 ± 0.05 mm and 0.81 ± 0.05 *vs* 0.57 ± 0.04 mm and 0.61 ± 0.03 mm, $P < 0.05$), CRP levels (1.47 ± 0.2 *vs* 0.75 ± 0.3 mg/dl, $P < 0.05$) and pulse pressure readings (57 ± 4 *vs* 43 ± 6 mmHg, $P < 0.05$). Serum adhesion molecules were elevated in plaque positive patients, but the difference was not significant.

Soluble E-selectin and sICAM-1 levels, but not sVCAM-1 levels, were positively correlated with serum phosphate and $Ca \times PO_4$ product (Figures 3 and 4). Patients with elevated $Ca \times PO_4$ product also showed significantly higher sE-selectin (209 ± 162 *vs* 104 ± 76 ng/ml, $P < 0.001$) and sICAM-1 levels (1096 ± 346 *vs* 802 ± 224 ng/ml, $P < 0.001$), but similar sVCAM-1 levels (952 ± 252 *vs* 819 ± 292 ng/ml, $P = 0.3$). Serum adhesion molecules showed no correlation with carotid artery measurements and CRP levels. Mean IMT and CBM(max) were significantly correlated with age ($r = 0.60$, $P = 0.000$ and $r = 0.58$, $P = 0.000$), number of plaques ($r = 0.53$, $P = 0.000$ and $r = 0.50$, $P = 0.000$) and pulse pressure ($r = 0.43$, $P = 0.001$ and $r = 0.43$, $P = 0.001$). Multiple regression analysis showed that age was the only independent predictor of mean IMT and CBM(max) measurements in haemodialysis patients ($P = 0.005$).

Discussion

This study has demonstrated two important findings. First is the well-known difficulty in achieving the NKF-K/DOQI targets for calcium, phosphate and $Ca \times PO_4$ product in haemodialysis patients. Pooling two random samples of prevalent US haemodialysis patients, Block *et al.* showed that approximately 60% of patients had phosphate levels above 5.5 mg/dl and almost 50% had $Ca \times PO_4$ product above 55 mg^2/dl^2 [2]. The dialysis outcomes and practices patterns study (DOPPS) in seven countries (France, Germany, Italy, Japan, Spain, UK and US) at two time points (1996–2001 and 2002–2004) has confirmed that the majority of the patients were beyond guideline ranges for mineral metabolism. Among patients outside the guideline range, 51.6–46.7% had serum phosphate >5.5 mg/dl, 50.1–48.6% had serum calcium >9.5 mg/dl and 43.4–38.6% had $Ca \times PO_4$ product >55 mg^2/dl^2. The DOPPS data showed that only 23–28% of the patients were within the guideline ranges for at least three measures [15]. A single centre study from the US has also proved the difficulty in achieving targets for phosphate (56% above 5.5 mg/dl), calcium (30% above 9.5 mg/dl) and $Ca \times PO_4$ product (43% above 55 mg^2/dl^2) [16]. In this study, 27.1% of the patients met all three targets and this was in accordance with DOPPS data where 23–28% of the patients had guideline ranges for at least three criteria. This group had better figures for hyperphosphataemia (31.8% above 5.5 mg/dl) and $Ca \times PO_4$ product (17.9% above), but not for hypercalcaemia (54.8% above 9.5 mg/dl). This may be related to the high percentage of calcium salt use as a phosphate binder (95% of the whole group) along with a significant amount of vitamin D use (29% of the whole group). High prevalence of hypercalcaemia has very important as increased 'calcium load' was recently proposed as the major pathogenic mechanism in the development of vascular calcification among chronic kidney disease (CKD) patients [5]. Recent studies proved that non-calcium based phosphate binders well controlled phosphate levels without the risk of hypercalcaemia and even decreased the progression of vascular calcification among haemodialysis patients compared to calcium salts [17–18].

The second and novel finding of this study is the potential association between disturbed calcium, phosphate and $Ca \times PO_4$ balance and an elevation in adhesion molecules. Following the landmark report of Block *et al.* [2], various reports confirmed the association between hyperphosphataemia, hypercalcaemia and elevated $Ca \times PO_4$ product and morbidity and mortality in maintenance haemodialysis patients [3,19–21]. In a recent study with the largest number of haemodialysis patients (more than 40.000), serum phosphate levels greater than 5 mg/dl, higher adjusted serum calcium concentrations and $Ca \times PO_4$ products above 45 mg^2/dl^2 were associated with an increased relative risk of death. The population attributable risk associated with disorders of mineral metabolism was 17.5%, owing largely to the high prevalence

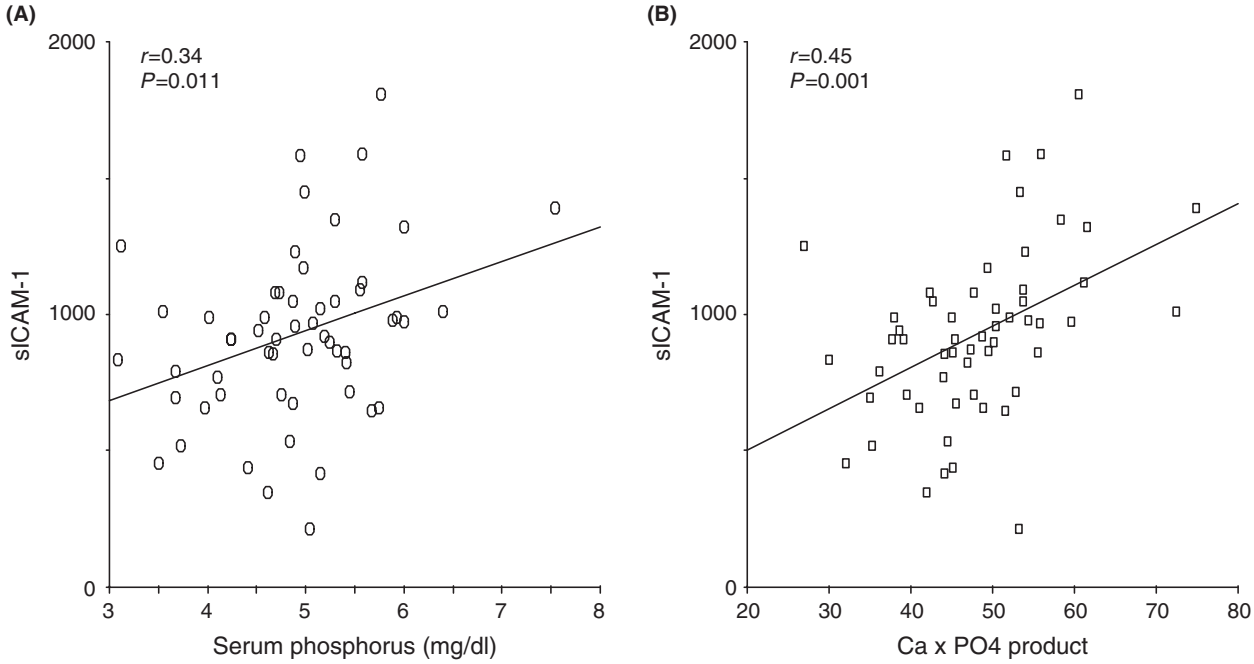

Fig. 3. Correlation between sICAM-1 and (A) serum phosphorus and (B) $Ca \times PO_4$ product.

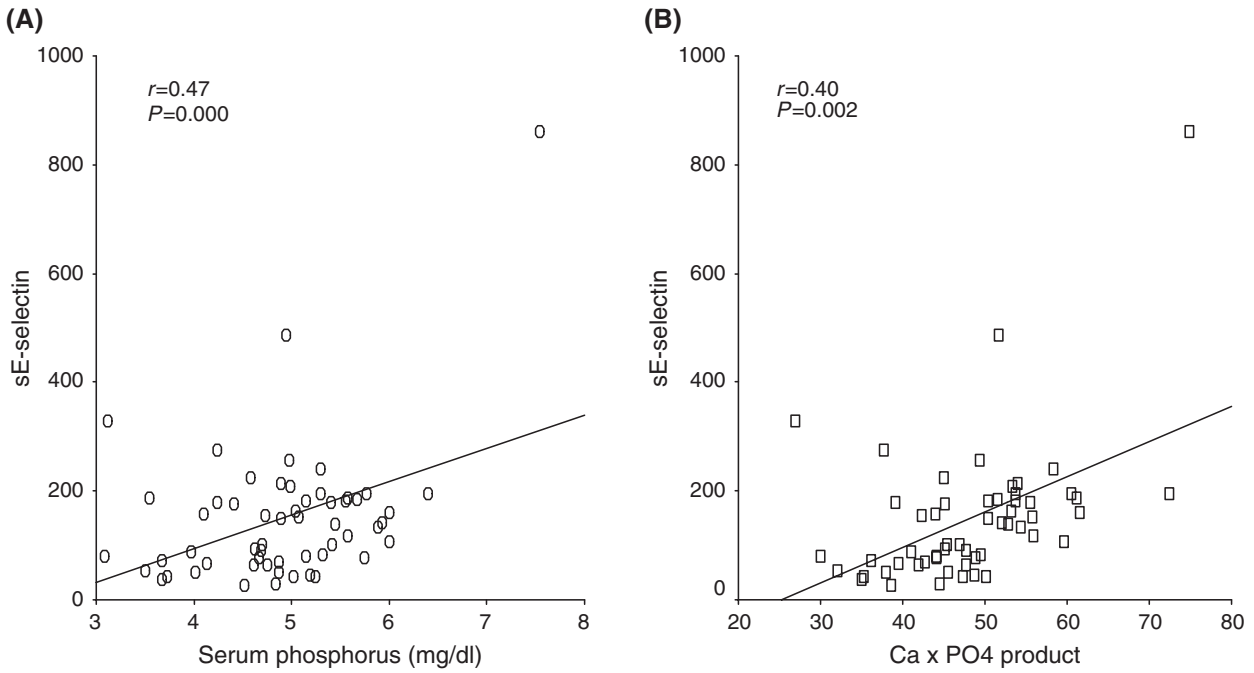

Fig. 4. Correlation between sE-selectin and (A) serum phosphorus and (B) $Ca \times PO_4$ product.

of hyperphosphataemia [20]. The mortality risk of elevated phosphate levels was recently extended to CKD patients, in whom each 0.5 mg/dl increase in serum phosphate levels was associated with a significant 35% increased risk for acute MI and a 28% increase risk for the combined end point of death plus nonfatal MI. This study has also shown that adjusted significant mortality risk increases when serum phosphate levels are above 3.5 mg/dl [22].

The exact pathogenesis linking abnormal mineral metabolism with extensive cardiovascular calcification and increased cardiovascular morbidity and mortality are still incompletely understood [5]. It is, however, clear that there will be no cardiovascular calcification without calcium and phosphate. Consistent with that, serum phosphate was able to stimulate phenotypic transformation of vascular smooth muscle cells into osteoblasts and to produce a pro-calcification milieu

in the vessel wall [23]. Such an environment accelerates the medial calcification and results in increased arterial stiffness, carotid tensile stress, aortic pulse wave velocity and all-cause mortality [8,19]. In a recent study with a large number of haemodialysis patients, hyperphosphataemia was also found to be a significant and independent risk factor for increased carotid IMT [24]. Several other reports showed a close association between coronary calcium scores and disturbed mineral metabolism [25–27]. We have also observed an increase in mean IMT and CBM(max) measurements in parallel to the deviation of mineral metabolism parameters from the target levels. Since carotid IMT and coronary calcium scores are accepted as non-invasive markers of atherosclerosis and predictors of vascular events, abnormal mineral metabolism may have a direct role in the natural process of atherosclerosis and cardiovascular disorders in the uraemic milieu.

In recent years it has become apparent that inflammation plays a central role in the development and progression of atherosclerosis [28]. Adhesion of circulating leukocytes to the endothelial cells and subsequent transendothelial migration is suggested as an important step in the formation and evolution of atherosclerotic lesions. Focal expression of ICAM-1, E-selectin and VCAM-1 has been demonstrated in human atherosclerotic plaques [29]. It was shown that uraemic atherosclerosis was preceded by upregulation of ICAM-1 expression in arterial endothelium and that formation of early lesions was accompanied by upregulation of VCAM-1 expression in the medial smooth muscle cell layer [30]. Soluble adhesion molecules have been found to predict carotid atherosclerosis and future cardiovascular events in the general population [13,31]. Serum levels of adhesion molecules have previously been reported to be elevated in patients with CKD [32–33]. Stenvinkel *et al.* showed that elevated sICAM-1 concentrations were an independent predictor of mortality in predialysis patients [34]. Papagianni *et al.* demonstrated that sICAM-1 was an independent predictor of carotid atherosclerosis [35] and increased sICAM-1 and SVCAM-1 levels were associated with vascular events [36] among haemodialysis patients.

The present data suggest that disturbed mineral metabolism may lead to an increase in serum adhesion molecules. In particular, sICAM-1 levels were significantly elevated in patients having hyperphosphataemia or hypercalcaemia and both sICAM and sE-selectin levels were elevated in patients with elevated $Ca \times PO_4$ product. This study has also shown that there is a near-linear increase in sE-selectin and sICAM-1 levels consistent with the degree of deviation from target levels. Highest sE-selectin and sICAM-1 levels were observed in patients who had all three parameters higher than the recommended targets. These findings may suggest that perturbed vessel wall due to hyperphosphataemia, hypercalcaemia and/or elevated $Ca \times PO_4$ product may be another 'nidus' for inflammatory stimuli, thus increase the release of adhesion molecules. This hypothesis was recently investigated in a small group of haemodialysis patients [37]. Movilli *et al.* demonstrated a significant hyperbolic correlation between $Ca \times PO_4$ and CRP levels and linear regression analysis showed a break-point at a $Ca \times PO_4$ of $55\,mg^2/dl^2$. They have also found that intensive lowering of mean $Ca \times PO_4$ from 62.8 to $46.3\,mg^2/dl^2$ significantly reduced the CRP levels [37]. In this study we have failed to find any association between abnormal mineral metabolism parameters and CRP. This may simply be caused by strict patient selection criteria of this study (i.e. comprising a relatively low-risk haemodialysis group with no evidence of vascular disease and diabetes) or insufficient statistical power to demonstrate an association. However, it is still tempting to speculate that abnormal mineral metabolism may activate inflammatory perturbations in the vessel wall along with other factors of uraemic milieu. This may accelerate the process of atherosclerosis and favour the development of cardiovascular changes and contribute to high cardiovascular morbidity and mortality in patients with abnormal mineral metabolism.

This study has several limitations, shortcomings and potential sources of error that merit consideration. The study was cross-sectional and observational in nature, the patient group was a 'small and selected' low-risk group (non-diabetics with no documented vascular disease) and adhesion molecule and carotid IMT measurements were done once and compared to time-averaged means of serum phosphate, calcium and $Ca \times PO_4$ product. The absence of significance for all adhesion molecules in different abnormalities of mineral metabolism or lack of correlations between mineral metabolism and carotid measurements may be related to the small number and low-risk status of the study group. Previous studies had non-selected patient groups and most significant effects were observed in high-risk patients, i.e. diabetics [24,36].

In conclusion, this study has shown the difficulty in controlling mineral metabolism and the association between abnormal mineral metabolism and an increase in adhesion molecules, mainly sICAM-1 and sE-selectin. Although further studies in larger patient groups are needed to elucidate and confirm the pathogenic implications of these findings, it may *still* generate a hypothesis linking deranged mineral metabolism with cardiovascular events. The presence of elevated adhesion molecules in a 'low-risk' patient group implies the urgent need for better control of mineral metabolism, in particular with agents that have potential anti-atherogenic and anti-inflammatory properties [18] and with efficient and more frequent dialysis.

Conflict of interest statement. None declared.

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Received for publication: 23.8.05
Accepted in revised form: 11.11.05