ORIGINAL ARTICLE

Excess Calcium, Magnesium and Phosphorus Deposition in the Epigastric Artery of Dialysis Patients Undergoing Renal Transplantation

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ABSTRACT

Objective: *Vascular calcification contributes to cardiovascular disease on dialysis patients. Arterial mineral content is modified but not well defined. We aim to define what is the concentration of calcium, magnesium and phosphorus in the epigastric artery of adult dialysis patients undergoing renal transplantation.*

Methods: *All renal allograft recipients who underwent surgery at our centre between May 2003 and December 2005 and consented to be taken small samples of epigastric artery were included in our cross-sectional study. Histological, radiological and spectrometric methods were used to measure vascular calcification, deposits and concentrations of calcium, phosphorus and magnesium in epigastric artery, which were correlated with clinical and biochemical characteristics. Mineral vascular content was compared with corresponding samples from cadaveric renal donors free from renal disease (control group).*

Results: *Calcium and magnesium concentrations in epigastric artery were much higher in recipients (n = 100) than in donors (n = 30). Histologically confirmed calcifications were more frequent in recipients. Calcium and magnesium content in epigastric artery were correlated directly with recipient age, pre-transplant serum P and Ca × P product. A high content of calcium and magnesium in this artery was observed in recipients with media and intimal calcification. Multivariate logistic regression showed that dialysis vintage > 3.5 years and calcium concentration in epigastric artery ≥ 4500 mg/kg wet weight were independent predictors of histological calcification.*

Conclusion: *Excess mineral deposition is observed in the epigastric artery of dialysis patients, where the recipient's age, serum P, Ca × P product and time on dialysis play a decisive role.*

Keywords: Dialysis, mineral metabolism, vascular calcification

INTRODUCTION

Mineral and bone disorders that accompany chronic kidney disease (CKD) are an important cause of cardiovascular mortality, not only on dialysis patients but also during earlier disease stages (1, 2). Cardiovascular disease originates from vascular calcifications (VCs), where classical vascular risk factors, age and inflammation, as well as dysregulated mineral metabolism, play an important role (3–7).

In clinical practice, VC diagnosis is based on imaging techniques (plain X-ray, spiral computed tomography and electron beam computed tomography for coronary arteries, cardiac magnetic resonance, echocardiography and ultrasound, and carotid artery ultrasound) (8, 9). However, before mineral deposits are detectable by imaging techniques or microscopy, alterations in the mineral content of the arterial wall appear. At present, no studies have investigated mineral concentrations in this tissue.

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To investigate the quantitative and qualitative mineral composition of the arterial wall in patients on dialysis, we collected samples of epigastric artery from recipients at the time of renal transplantation and compared it with that of cadaveric renal donors, taken here as a control group.

SUBJECTS AND METHODS

Participants

This study includes all renal allograft recipients who underwent surgery at the Hospital Virgen de las Nieves in Granada, Spain, between May 2003 and December 2005. All patients provided their informed consent to participate and donate a sample of epigastric artery during the operation. The study was conducted according to the principles of the Declaration of Helsinki and in compliance with the *International Conference on Harmonization/Good Clinical Practice* regulations. Oversight was provided by the Research Ethics Committee of the University of Granada, which reviewed and approved the protocol. Participants were patients on dialysis receiving renal transplantation and age-matched cadaveric renal donors served as a control group.

Radiological study of calcifications

Calcifications were scored with the Adragao scoring system for the hands (range 0–4) and pelvis (range 0–4), and the Kauppila index (range 0–24) for the aorta (10, 11). A single observer recorded all evaluations.

Sample preparation and processing

Samples of epigastric artery were obtained in the course of allograft surgery or the surgical removal of the donated kidney. Arterial samples were divided into two portions: one was used to quantify Ca, P and Mg per unit weight with atomic absorption spectrophotometry, and the other one was used for histological study (haematoxylin and eosin, trichromic, von Kossa and orcein staining).

Histological study of calcifications

Artery samples were fixed immediately after excision in 10% buffered formalin (Leica, Barcelona, Spain) for 24 hours. Transverse sections were cut and embedded in paraffin with an automated tissue processing system (Shandon Pathcentre, Thermo Shandon, Pittsburgh, PA, USA). Histological sections of 3 μm were cut, deparaffinated, hydrated and stained with haematoxylin–eosin and the von Kossa silver impregnation technique for Ca deposits (12). A single observer recorded the presence of Ca deposits, their location and number.

Mineral concentration in the arterial wall

The samples were refrigerated between 4°C and 8°C, and then placed in 2% vol/vol NO3H (Merck, Darmstadt, Germany) for 24 hours and rinsed in double-distilled water (Millipore, Bedford, MA, USA) to avoid chemical contamination. Fat was removed before mineral concentrations were quantified. To determine dry weight, samples were heated to 100°C until constant weight was attained and placed in a beaker covered with an inverted watch glass. Over a hot sand bath, 2 mL 65% vol/vol nitric acid (Merck, Darmstadt, Germany) was added, followed by 2 mL 60% perchloric acid (Merck). After the solution cooled down, 1 mL 5N hydrochloric acid (Merck) was added, and finally double-distilled water (MilliQ) was added to a total volume of 10 mL.

To determine Ca and Mg concentrations, a Perkin Elmer AAnalyst 300 atomic absorption spectrophotometer with single-element Lumina lamps (PerkinElmer, Rodgau, Germany) was used. Flame absorption was used with an air–acetylene mixture with 1% lanthanum chloride to inhibit interference from other chelators. To determine P concentrations, an Uvikon-XS UV/V spectrophotometer with the Fiske–Subbarow method based on ammonium molybdate was used.

All reagents were of a high purity grade (Merck, Madrid, Spain). Water was double distilled with a Milli-Q system (Millipore, Madrid, Spain). The standards for Ca and Mg were from PerkinElmer, and the standard for P was from Química Clínica Aplicada (Amposta, Spain). The reference material used for quality control in all mineral concentration assays was CRM 185 (Community Bureau of Reference, Brussels, Belgium). Before the assays, the quality of the analytic and methodological processes was checked by generating a standard curve for each mineral from samples of the reference material tested in quadruplicate and samples of arterial tissue tested in triplicate, with a 95% confidence interval (CI).

Statistical analysis

The sample size of 100 recipients permitted a precision of ± 20% in the estimated mean values of mineral concentration in the arterial wall, assuming a standard error similar to the mean. In the donor group, these values were expected to be distributed normally and to show lower dispersion than that of the recipient group. Accordingly, a standard deviation of 60% of the mean was assumed, and a sample size of 30 was needed to estimate mineral concentrations with 20% precision. In the recipient group, mineral concentrations were expected to be more than twice as high as in the control

group, so the sample size of 30 donors was needed to detect differences between groups at a significance level of $\alpha = 0.05$.

The normal distribution of quantitative variables was verified. Descriptive statistics for quantitative variables were reported as the mean ± SD for variables distributed normally, and as the median and interquartile range (P25 and P75) for those that were not distributed normally. For categorical variables, the number of valid observations and percentage distribution in each category were reported. To study associations between variables, Student's *t* test was used if one variable was a two-level categorical variable and the other was a quantitative variable that was distributed normally. For variables that were not normally distributed, we used Mann–Whitney's *U* test. Associations between discrete quantitative variables or variables that were not distributed normally were determined with Spearman's correlation coefficient.

Univariate logistic regression analysis was used to determine the association between the presence of histologically confirmed calcifications and the independent variables sex, time on dialysis, mean pre-transplant serum values of Ca, P, Ca × P product, intact parathyroid hormone, alkaline phosphatase and lipid profile recorded during the year before transplantation, and ion concentration per kg wet weight and per kg dry weight in the arterial wall. The biochemical profile was measured using biochemical methods of clinical practice (13). For these analyses, continuous variables were stratified into two levels with the 70th percentile as the cut-off value. For multivariate regression analysis, logistic regression models were fitted with the variables that showed a significant association ($p < 0.05$). Two-tailed hypothesis tests were used with a significance level of $\alpha = 0.05$. All studies were performed using the statistical package SPSS 20.0.

RESULTS

Baseline characteristics of recipients and donors: Biochemical profile in recipients

We studied 130 individuals, 100 recipients and 30 cadaveric renal donors with no renal disease. Baseline characteristics were similar between both groups. Men were recorded in 65% of recipients and in 50% of donors. Mean age was 46 ± 14.6 years in recipients and 44 ± 16.1 years in donors. Low or normal body mass index (BMI < 25 kg/m^2) was recorded in 49% of recipients and 50% of donors. Overweight (BMI > 25–30 kg/m^2) was present in 39% of recipients and 33% of donors. Obesity (BMI > 30 kg/m^2) was recorded in 12% of recipients and 17% of donors.

Donors

The most frequent cause of death was head trauma (50%), followed by stroke (36.7%). A total of 26.7% suffered from hypertension, 2% from diabetes, 2% from ischemic heart disease and 1% from peripheral valve disease; 17.9% of donors were smokers. Mean serum creatinine was 1.0 ± 0.63 mg/dL.

Recipients

The most frequent cause of CKD was glomerular disease (32%), followed by vascular disease (17%), interstitial disease (12%) and diabetes (5%); the 39.2% of them were smokers. Before transplantation, 84% had received haemodialysis, 7% had received peritoneal dialysis and 9% had received both types of therapies; 61% had received dialysis for more than 2 years prior to transplantation, with a mean time on dialysis of 2.6 years (1.6–4.1). Mean pre-transplant values for laboratory parameters in recipients are shown in Table 1. All variables showed a normal distribution.

Table 1: Biochemical profile in renal allograft recipients

Parameter	N	Mean ± SD
Calcium (mg/dL)	100	9.4 ± 0.83
Phosphorus (mg/dL)	100	5.6 ± 1.44
Ca x P	100	52.7 ± 13.11
iPTH (pg/mL)	100	307 ± 262.0
ALP (IU/L)	96	95.9 ± 50.42
Total cholesterol (mg/dL)	96	155 ± 39.8
LDL-c (mg/dL)	92	80 ± 29.6
HDL-c (mg/dL)	91	46 ± 14.4
Triglycerides (mg/dL)	94	151 ± 74.3
Fibrinogen (mg/dL)	49	401 ± 117.6

N = number of observations for each parameter; iPTH = intact parathyroid hormone; ALP = alkaline phosphatase; LDL-c = low-density lipoprotein cholesterol; HDL-c = high-density lipoprotein cholesterol. Represented are the mean serum values of the 12 months preceding transplantation.

Relationship between mineral content and histological calcification in epigastric artery

Global mineral values in epigastric artery samples from both recipients and donors are shown in the upper part of Table 2. These data did not show the normal distribution. Calcium and magnesium concentrations were significantly higher in recipients than those in donors, both in wet- and dry weight. Phosphorus concentration was also higher in recipients, although the difference

compared with donors was statistically significant only for wet weight ($p < 0.05$).

The middle and lower parts of Table 2 show the comparisons of mineral content in epigastric artery between groups according to the presence of histological calcifications. Recipient samples with calcifications and no calcifications showed a Ca concentration per unit of wet weight higher than donors ($p < 0.05$).

Vascular calcification measured by histological and X-ray methods

Histologic calcifications in any layer of the epigastric artery were significantly more frequent in recipients than in donors (63.0% *vs* 25.0%, $p < 0.01$). The difference between groups remained statistically significant when comparing both intima (21.9% *vs* 0%, $p < 0.01$) or media (57.6% *vs* 21.4%, $p < 0.01$) locations separately.

Table 2: Mineral content in epigastric artery from recipients (R) (n = 100) and donors (D) (n = 30): relationship with histological calcification

All individuals						
		Mean	**Median**	**SD**	**P25**	**P75**
Ca (mg/kg wet weight)**	Donor	1481	885	1603	535	1277
	Recipient	16 215	1946	36 589	1289	6426
Ca (mg/kg dry weight)**	Donor	3471	2647	2827	1537	4214
	Recipient	34 735	4849	78 341	2736	12 991
Mg (mg/kg wet weight)**	Donor	185	130	180	86	235
	Recipient	431	200	642	148	426
Mg (mg/kg dry weight)*	Donor	438	387	295	242	550
	Recipient	852	489	1083	297	788
P (mg/kg wet weight)*	Donor	1424	758	1304	513	2113
	Recipient	5350	1633	8294	561	7502
P (mg/kg dry weight)	Donor	5558	2659	6222	954	9545
	Recipient	9723	3438	14 595	1297	13 621
Patients with histologic epigastric artery calcification (D, 25.9%; R, 64.5%)						
		Mean	**Median**	**SD**	**P25**	**P75**
Ca (mg/kg wet weight)**	Donor	1786	743	1977	641	4657
	Recipient	24 003	3176	43 547	1536	25 344
Ca (mg/kg dry weight)	Donor	4162	2766	3491	1521	6668
	Recipient	51 443	6021	93 628	2855	69 834
Mg (mg/kg wet weight)	Donor	270	151	306	80	411
	Recipient	564	285	763	167	552
Mg (mg/kg dry weight)	Donor	492	430	271	318	589
	Recipient	1083	563	1288	323	1391
P (mg/kg wet weight)	Donor	1613	1106	1272	533	2683
	Recipient	6227	2235	9454	857	7656
P (mg/kg dry weight)	Donor	5663	2804	6437	1054	13 333
	Recipient	11 035	4141	16 576	1706	14 000
Patients without histologic epigastric artery calcification (D, 74.1%; R, 35.5%)						
		Mean	**Median**	**SD**	**P25**	**P75**
Ca (mg/kg wet weight)*	Donor	1436	911	1516	535	1277
	Recipient	1750	1548	1153	1109	1951
Ca (mg/kg dry weight)	Donor	3381	2524	2615	1608	4214
	Recipient	4303	3809	2919	2707	5637
Mg (mg/kg wet weight)	Donor	159	130	112	87	235
	Recipient	184	159	96	135	184
Mg (mg/kg dry weight)	Donor	432	387	310	225	550
	Recipient	430	420	171	295	561
P (mg/kg wet weight)	Donor	1359	646	1374	482	1991
	Recipient	3722	1164	5316	436	6743
P (mg/kg dry weight)	Donor	5673	2783	6437	891	9545
	Recipient	7335	2861	9866	1101	12 239

P25 = 25th percentile; P75 = 75th percentile. Mann–Whitney *U* test: * = $p < 0.05$; ** = $p < 0.01$.

No differences were shown when the calcification was located in the internal elastic (7.5% *vs* 7.1%) or adventitia (5.2% *vs* 0%) laminas. The frequency of intimal hyperplasia in both groups did not differ (46% *vs* 28%).

In recipients, X-ray studies showed evidence of calcification in 66.7%, of which 54.9% were located in the aorta and 55.9% in the iliac arteries; 17% of recipients showed radiologic calcifications in hands, 20.2% suffered from subperiosteal resorption in fingers and 17.1% showed salt-and-pepper resorption in cranium.

Relationship between mineral content in epigastric artery according to baseline characteristics and histological calcification in recipients

A significant direct correlation between the recipient age and some minerals in the arterial wall (such as Ca ($r = 0.22$, $p < 0.05$) and Mg content ($r = 0.24$, $p < 0.05$) per unit wet weight) was observed. Serum P before transplantation correlated significantly with Ca content ($r = 0.26$ per unit wet weight, and $r = 0.31$ per unit dry weight, $p < 0.05$ in both), and with Mg content per unit wet weight ($r = 0.27$, $p < 0.05$). The Ca × P product correlated significantly with Ca content per unit wet ($r = 0.28$, $p < 0.01$) and dry weight ($r = 0.30$, $p < 0.01$), and with Mg content per unit wet ($r = 0.22$, $p < 0.05$) and dry weight ($r = 0.25$, $p < 0.05$).

Table 3 shows comparisons between mineral content according to the presence/absence of media calcification as well as intimal hyperplasia of epigastric artery. Recipients with media calcification showed higher Ca content per unit wet and dry weight and higher Mg content per unit wet and dry weight. Likewise, recipients with intimal hyperplasia showed higher Ca and Mg content per unit wet and dry weight.

Diabetic recipients showed higher Ca concentrations in epigastric artery per unit wet and dry weight than recipients without diabetes ($p < 0.05$). The median Ca concentration per unit wet weight (referred to percentiles P25–P75) was 7.30 mg/kg (CI: 3.16, 103.18) in diabetic patients and 1.85 mg/kg (CI: 1.28, 5.44) in non-diabetic patients. The median concentration per unit dry weight was 17.12 mg/kg (CI: 7.81, 192.63) in recipients with diabetes *versus* 4.49 mg/kg (CI: 2.73, 9.84) in recipients without diabetes. There were no significant differences between recipients with and without diabetes regarding any other mineral concentrations.

The results of the univariate logistic regression analysis are showed in Table 4. Histologic calcifications in epigastric artery were associated with preceding time on dialysis (vintage), mean Ca pre-transplantation concentration, low-density lipoprotein cholesterol, and Ca and Mg concentration in the arterial wall. In the multivariate models (Table 4), recipients whose dialysis vintage was < 3.5 years (compared with ≥ 3.5 years) and those whose arterial wall Ca concentration was < 4500 mg/kg wet weight (compared with ≥ 4500 mg/kg) had a higher risk of histologic calcifications.

Table 3: Comparisons of mineral content in epigastric artery wall according to histologic study in recipients (n = 100)

	Media calcification			
	No (41.8%)		**Yes (58.2%)**	
	Mean ± SD	**P25-P75**	**Mean ± SD**	**P25-P75**
Ca (mg/kg wet weight)**	2092 ± 1590	1166–2357	26 684 ± 45 660	1619–32 621
Ca (mg/kg dry weight)**	5035 ± 3757	2755–6697	57 260 ± 98 303	2795–79 944
Mg (mg/kg wet weight)**	195 ± 102	137–200	607 ± 801	184–605
Mg (mg/kg dry weight)*	452 ± 185	319–578	1159 ± 1352	310–1518
P (mg/kg wet weight)	3909 ± 5316	497–7122	6422 ± 9895	838–7701
P (mg/kg dry weight)	7656 ± 9744	1295–13 602	11 306 ± 17 395	1483–13 810
	Intimal calcification			
	No (54.4%)		**Yes (45.6%)**	
	Mean ± SD	**P25-P75**	**Mean ± SD**	**P25-P75**
Ca (mg/kg wet weight)*	10 741 ± 1978	1264–2865	22 576 ± 40 835	1534–24 986
Ca (mg/kg dry weight)*	22 457 ± 62 396	2732–7511	49 402 ± 92 731	3295–64 284
Mg (mg/kg wet weight)*	292 ± 326	146–275	592 ± 855	156–547
Mg (mg/kg dry weight)*	628 ± 607	293–654	1119 ± 1426	339–1373
P (mg/kg wet weight)	4925 ± 7727	517–7115	5844 ± 8992	970–7694
P (mg/kg dry weight)	9504 ± 14 290	1063–14 348	9986 ± 15 150	1979–13 448

% = Percentage of observations; P25 = 25th percentile; P75 = 75th percentile. Mann–Whitney *U* test: * = $p < 0.05$; ** = $p < 0.01$.

Table 4: Logistic regression analysis of factors associated with histologic calcifications in epigastric artery (n = 100)

	Univariate analysis			Multivariate analysis		
Independent variables	**OR**	**95% CI**	***P***	**OR**	**95% CI**	***P***
Clinical antecedents						
Time on dialysis (< 3.5 *vs* ≥ 3.5 years)	3.4	(1.2–9.3)	< 0.05	4.2	(1.1–15.7)	< 0.05
Pre-transplant serum Ca (< 9.82 *vs* ≥ 9.82 mg/dL)	3.0	(1.1–8.2)	< 0.05			0.2
Pre-transplant LDL cholesterol (≥ 100 *vs* < 70 mg/dL)	3.4	(1.1–11.1)	< 0.05			0.2
Mineral content in epigastric artery (mg/kg wet weight)						
Calcium (< 4500 *vs* ≥ 4500 mg/kg)	9.5	(2.0–44.5)	< 0.01	9.4	(1.9–47.2)	< 0.01
Magnesium (< 350 *vs* ≥ 350 mg/kg)	3.7	(1.1–12.4)	< 0.05			0.2

The following independent variables were not significantly associated with the presence of histologic calcifications in univariate analysis: sex, pre-transplant serum P, Ca x P, iPTH, ALP and HDL cholesterol, phosphorus content in epigastric artery.

DISCUSSION

To the best of our knowledge, this is the first study that provides a quantitative analysis of Ca, P and Mg in the vascular wall of long-term adult dialysis patients. We found that these individuals show much higher Ca and Mg content in their epigastric arteries than donors of the same age. Old recipient age, pre-transplantation serum P and Ca × P products are determinants of the Ca and Mg content. Finally, we also found that the odds of clinically evident histological calcification in the epigastric artery wall of dialysis patients increases with dialysis vintage and with excess Ca concentration in the arterial wall.

Our results are concordant with a recent study performed on children (14), where medium-sized muscular arteries were removed at omentectomy during a peritoneal dialysis catheter insertion or at renal transplantation from 10 pre-dialysis and 24 dialysis children. Mesenteric arteries removed at planned intra-abdominal surgery from six children of similar age served as controls. Children with CKD showed higher Ca load in the vascular wall than controls, and dialysis children also exhibited higher Ca load than pre-dialysis ones. Altogether, these observations expand previous studies regarding quantitative evidence that calcium accumulation in the vascular wall starts already in the pre-dialysis phase (15–17) and that factors related to the dialysis further trigger this condition (18, 19). Because our study assessed Ca content with a different methodology than the preceding study (atomic absorption spectrophotometry instead of cresolphthalein complexone chemistry (14)), results cannot be compared quantitatively, but both evidence the excess Ca deposition in the arterial wall that occurs in end-stage renal disease as well as in dialysis.

More frequently, we found histologically confirmed calcifications in epigastric artery from recipients, and its location in intima and media is consistent with earlier studies (20–23). When these calcifications were present, elevated Ca and Mg concentrations were also observed. Such results are coherent with those of Yang *et al* (24), pinpointing towards the important role of calcium on the development of VC by stimulating mineralization in the presence of normal phosphorus concentrations, and may suggest that when phosphorus concentrations are elevated, Ca deposition is accelerated synergistically. However, we do not have a clear explanation about the higher Mg concentration we found out in the vessel wall. It is known Mg abnormalities in CKD. Recently, an inverse relationship between serum magnesium concentrations and VC has been reported, and lower serum Mg levels are related to increased arterial calcification in haemodialysis patients (25, 26). Taken together all these pieces of evidence, if we consider Mg a natural calcium antagonist, we can hypothesize that the higher Mg content in the vascular wall might be trying to inhibit VC. This will have to be settled in further investigations.

Vascular calcification related to CKD is a high-regulated cell-mediated process with many similarities to bone formation and is associated with many risk factors (*eg* ageing, hypertension, diabetes and dyslipidemia) as well as hyperphosphatemia, extreme hyper-hypoparathyroidism and administration of excess calcium salts as well as vitamin D (27–31). In our study, the main factors that were associated with VC in dialysis patients were age, diabetes, serum P and Ca × P product, all of which were correlated directly with Ca and Mg content in the arterial wall, although not with the arterial P content. In our study, against our expectations, we did not find a relationship between VC and serum Ca, obesity, dyslipidaemia or PTH levels (32).

In summary, our study evidences the existence of excess Ca, Mg and P content in the artery wall of dialysis patients and reports its main clinical determinants in, so far, the largest and best characterized cohort available of this kind. A limitation in the interpretation of our

findings is that our control group consisted of cadaveric renal donors, which may not be exempted from other diseases. Because, as per study design, we only included dialysis patients eligible for transplantation, we acknowledge a selection of potentially healthier patients. However, both limitations can only underestimate the differences here reported.

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