Effect of a magnesium-based phosphate binder on medial calcification in a rat model of uremia

Tineke M. De Schutter1, Geert J. Behets1, Hilde Geryl1, Mirjam E. Peter2, Sonja Steppan2, Kristina Gundlach2, Jutta Passlick-Deetjen3, Patrick C. D’Haese1 and Ellen Neven1

1Laboratory of Pathophysiology, Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium; 2Fresenius Medical Care Deutschland GmbH, Bad Homburg, Germany and 3Department of Nephrology, University of Dusseldorf, Dusseldorf, Germany

Calcium-based phosphate binders are used to control hyperphosphatemia; however, they promote hypercalcemia and may accelerate aortic calcification. Here we compared the effect of a phosphate binder containing calcium acetate and magnesium carbonate (CaMg) to that of sevelamer carbonate on the development of medial calcification in rats with chronic renal failure induced by an adenine diet for 4 weeks. After 1 week, rats with chronic renal failure were treated with vehicle, 375 or 750 mg/kg CaMg, or 750 mg/kg sevelamer by daily gavage for 5 weeks. Renal function was significantly impaired in all groups. Vehicle-treated rats with chronic renal failure developed severe hyperphosphatemia, but this was controlled in treated groups, particularly by CaMg. Neither CaMg nor sevelamer increased serum calcium ion levels. Induction of chronic renal failure significantly increased serum PTH, dose-dependently prevented by CaMg but not sevelamer. The aortic calcium content was significantly reduced by CaMg but not by sevelamer. The percent calcified area of the aorta was significantly lower than vehicle-treated animals for all three groups. The presence of aortic calcification was associated with increased sox9, bmp-2, and matrix gla protein expression, but this did not differ in the treatment groups. Calcium content in the carotid artery was lower with sevelamer than with CaMg but that in the femoral artery did not differ between groups. Thus, treatment with either CaMg or sevelamer effectively controlled serum phosphate levels in CRF rats and reduced aortic calcification.

Correspondence: Patrick C. D’Haese, Laboratory of Pathophysiology, University of Antwerp, Campus Drie Eiken, Building T.0, Universiteitsplein 1, Antwerp, B-2610, Belgium. E-mail: Patrick.dhaese@ua.ac.be

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Increased serum phosphate concentrations play a role in transdifferentiation of vascular smooth muscle cells toward osteochondrogenic cells that lead to mineralization of the tunica media of large blood vessels in patients with chronic kidney disease. This biological and bone-resembling process is now well recognized as an important mechanism of medial calcification in chronic renal failure (CRF), a pathological process that ultimately leads to reduced elasticity of the blood vessel wall, increased systolic blood pressure, and left ventricle hypertrophy. Approximately 50% of the mortality in hemodialysis patients is a direct consequence of cardiovascular disease, of which medial calcification is a major risk factor. Phosphate binders are administered to control serum phosphorus levels in end-stage renal disease patients, particularly when treated by dialysis. Phosphate binders based on calcium are rather inexpensive and thus frequently used. In addition, it was shown in an animal model that they—probably because of their phosphate-binding capacity—also slow the progression of vascular calcification as compared with untreated CRF animals. However, compared with non-calcium-based phosphate binders such as lanthanum carbonate (Fosrenol), sevelamer carbonate (Renvela), and sevelamer hydrochloride (Renagel), treatment with calcium-based agents may lead to an increased number of hypercalcemic episodes, which may promote development of vascular calcification. Hence, both hyperphosphatemia and hypercalcemia may contribute to vascular calcification. Another alternative phosphate-binding agent is the calcium acetate/magnesium carbonate–containing phosphate binder (OsvaRen, hereafter named CaMg). The safety and efficacy of this and other magnesium-based phosphate binders has already been demonstrated in clinical studies, but their potential to prevent the development and/or progression rate of vascular calcification has not yet been investigated, although in vitro studies have already shown a beneficial effect of magnesium on the deposition of calcium and an inhibiting effect on the osteogenic differentiation of cultured vascular smooth muscle cells.

In addition, data from clinical studies in dialysis patients demonstrated that low serum magnesium concentrations are associated with progression of vascular calcification and...
increased mortality. Higher serum magnesium levels have been reported to protect against the development and progression of vascular calcification, although the mechanisms underlying this effect are rather unknown.

In this study, the effect of the magnesium-based phosphate binder CaMg on the development of vascular calcification in experimental CRF was investigated and compared with that of sevelamer carbonate with equal amounts of active salt (for details see ‘Materials and Methods’).

RESULTS

Rats were fed a 0.75% adenine/2.5% protein diet to induce renal failure. After 1 week, treatment with phosphate binders or vehicle was started until the time of killing at week 6 (Figure 1). There was limited mortality: one animal from each treatment group died 1 day before the planned day of killing. No statistically significant differences between groups were seen for body weight and water and food consumption (data not shown).

Biochemical analyses

Results of the biochemical analyses of serum and urine samples are listed in Table 1 and Figure 2. A pronounced renal failure was induced in all groups. Renal function decreased significantly after 4 weeks of adenine/low protein treatment as indicated by the increase in serum creatinine concentrations, which were comparable between all groups during the whole study period (Table 1). Hyperphosphatemia developed in vehicle-treated rats and reached levels of up to 20 mg/dl after 4 weeks of CRF (Table 1 and Figure 2a). At this time point, treatment with 750 mg/kg CaMg already significantly reduced serum phosphorus levels, whereas this reduction occurred only at week 6 for the sevelamer-treated group. Urinary phosphate levels decreased in both CaMg-treated groups from week 4 onwards, whereas sevelamer lowered phosphate concentrations in the urine only at the end of the study (Table 1 and Figure 2b). After 4 and 6 weeks of CRF, serum intact fibroblast growth factor 23 (iFGF-23) levels were dramatically increased in all groups; however, phosphate binder treatment could not reduce iFGF-23 concentration in the circulation (Table 1). Total serum calcium levels significantly increased in all phosphate binder-treated groups compared with week 0 as well as in CaMg-treated groups at the end of the study compared with vehicle-treated animals (Table 1). However, ionized calcium levels significantly decreased throughout the study in all groups and no significant difference could be found between groups at any time point (Table 1 and Figure 2c). Urinary excretion of calcium was significantly increased in all groups as compared with baseline values (Table 1). As could be expected, serum magnesium concentrations dose-dependently increased during the study in CRF groups treated with CaMg (Table 1). Induction of CRF went along with a significant increase in serum parathyroid hormone (PTH) concentrations. Treatment with CaMg dose-dependently suppressed this increase whereas sevelamer did not (Table 1).

Figure 1 | Study setup. CaMg, calcium acetate/magnesium carbonate; CaMg375, 375 mg/kg CaMg; CaMg750, 750 mg/kg CaMg; Sev750, 750 mg/kg sevelamer.

Vascular calcification

After 6 weeks of CRF, vehicle-treated CRF animals developed severe calcification in the aorta, femoral and carotid arteries (aortic calcium content of rats with normal renal function 0.35 ± 0.40 mg per g wet tissue), as shown in Figure 3a representing the aortic calcium content. Treatment with both 750 mg/kg CaMg (CaMg750) and 375 mg/kg CaMg (CaMg375) significantly reduced the aortic calcium content as compared with vehicle-treated CRF animals, whereas no significant effect could be observed for sevelamer. Histological analysis of von Kossa-stained sections of the aorta showed a significantly reduced percent area of calcification in the CaMg-treated animals as well as in the sevelamer-treated group (Figure 3b). Treatment with sevelamer carbonate, but not with CaMg, was associated with significant lower tissue calcium content in the carotid artery. There was no significant difference at all between CRF groups regarding calcium content of the femoral artery.

Although no significant difference in the magnesium content of the aorta could be found between groups, magnesium content differed in the CaMg750 group for carotid artery (increased) and in Sev750 for femoral artery (decreased) compared with vehicle (Table 2). The Mg/Ca ratio increased in all phosphate binder-treated groups, but this should be interpreted in light of the reduced calcium deposition in the vessels of these animals.

Synchrotron-based X-ray diffraction analysis of the aortic calcifications in CaMg-, sevelamer- and vehicle-treated animals indicated mineral precipitates of the various groups to exclusively consist of calcium hydroxyapatite (Figure 4). None of the samples contained whitlockite or any other type of calcium phosphate mineral.

Gene expression analyses

Figure 5 illustrates the expression of osteochondrogenic genes in the aorta. The expression of bmp-2 (bone morphogenetic protein 2), the chondrocyte-specific transcription factor sox9 (sex determining region Y-box 9), and the calcification inhibitor mgp (matrix gla protein) was significantly increased in CRF rats compared with rats with normal renal function. Interestingly, in CRF animals the expression of bmp-2, sox9, and mgp was clearly associated with the presence of vascular calcification.
calcification in the aorta. Despite a reduction of calcification, there was no difference in the expression pattern of these genes in the phosphate binder groups compared with vehicle or between the different phosphate binder groups. There was no change in the expression of osterix (cbfa-1) and the magnesium transporter TRPM7

| Table 1 | Serum and urine biochemistry |
|---------|-----------------------------|
|         | Weeks of CRF                 |
|         | Baseline (n = 14)            |
|         | 0 (n = 14)                  |
|         | 1 (n = 7)                   |
|         | 4 (n = 7)                   |
|         | 6 (n = 14)                  |
| **Serum creatinine (mg/dl)** |                          |
| Vehicle | 0.28 ± 0.02                 |
| CaMg750 | 0.27 ± 0.04                 |
| CaMg375 | 0.28 ± 0.04                 |
| Sev750  | 0.29 ± 0.03                 |
| **Serum P (mg/dl)**              |
| Vehicle | 7.01 ± 0.78                 |
| CaMg750 | 6.45 ± 0.81                 |
| CaMg375 | 6.73 ± 0.83                 |
| Sev750  | 6.75 ± 0.78                 |
| **Serum Ca (mg/dl)**             |
| Vehicle | 10.49 ± 0.23                |
| CaMg750 | 10.19 ± 0.21*               |
| CaMg375 | 10.49 ± 0.25                |
| Sev750  | 10.68 ± 0.18**              |
| **Serum Mg (mg/dl)**             |
| Vehicle | 1.95 ± 0.08<sup>±</sup>     |
| CaMg750 | 2.00 ± 0.13<sup>±</sup>     |
| CaMg375 | 1.90 ± 0.14<sup>±</sup>     |
| Sev750  | 1.85 ± 0.12<sup>±</sup>     |
| **Serum Ca<sup>2+</sup> (mg/dl)** |
| Vehicle | 5.26 ± 0.13                 |
| CaMg750 | 5.32 ± 0.10                 |
| CaMg375 | 5.29 ± 0.16                 |
| Sev750  | 5.32 ± 0.09                 |
| **Serum PTH (pg/ml)**           |
| Vehicle | 213 ± 1.876                 |
| CaMg750 | 209.7 ± 148.9               |
| CaMg375 | 164.4 ± 48.5                |
| Sev750  | 198.1 ± 67.0                |
| **Serum iFGF-23 (ng/ml)**       |
| Vehicle | 0.30 ± 0.05                 |
| CaMg750 | 0.32 ± 0.09                 |
| CaMg375 | 0.28 ± 0.09                 |
| Sev750  | 0.30 ± 0.09                 |
| **Serum pH**                     |
| Vehicle | 7.39 ± 0.04                 |
| CaMg750 | 7.39 ± 0.06                 |
| CaMg375 | 7.42 ± 0.03                 |
| Sev750  | 7.40 ± 0.05                 |
| **Serum HCO<sub>3</sub> (mg/dl)** |
| Vehicle | 25.6 ± 1.0                  |
| CaMg750 | 25.1 ± 0.9                  |
| CaMg375 | 26.4 ± 1.7                  |
| Sev750  | 25.3 ± 1.6                  |
| **Urinary P (mg/g creatinine)**  |
| Vehicle | 8.45 ± 2.68                 |
| CaMg750 | 7.66 ± 1.61                 |
| CaMg375 | 7.04 ± 1.67                 |
| Sev750  | 7.14 ± 2.07                 |
| **Urinary Ca (mg/g creatinine)** |
| Vehicle | 1.45 ± 0.38<sup>±</sup>     |
| CaMg750 | 1.62 ± 0.74                 |
| CaMg375 | 1.65 ± 0.88                 |
| Sev750  | 1.59 ± 0.90                 |

Abbreviations: CaMg, calcium acetate/magnesium carbonate; CaMg375, CaMg 375 mg/kg; CaMg750, CaMg 750 mg/kg; CRF, chronic renal failure; iFGF-23, intact fibroblast growth factor 23; PTH, parathyroid hormone; Sev, sevelamer; Sev750, sevelamer carbonate 750 mg/kg.

*Significant difference versus vehicle at the same time point.

Significant difference versus week 0 of the same group.

Biochemical parameters are measured in serum and urine.

Data are expressed as mean ± s.d.
(transient receptor potential cation channel, subfamily M, member 7) between CRF and normal renal function groups, and there was no difference in the expression of these genes between calcified and noncalcified vessels.

DISCUSSION
Based on clinical findings in dialysis patients, it has been demonstrated that patients with reduced serum magnesium concentrations have more arterial calcifications compared with patients with normal or increased serum magnesium values. In addition, low serum magnesium concentrations are associated with increased vascular stiffness and increased mortality (Passlick-Deetjen et al., abstract OM026 presented at ERA-EDTA 2010; Lacson et al., abstract F-PO1488 presented at ASN 2009) whereas increased serum magnesium improves intima media thickness and pulse wave velocity in chronic kidney disease patients.

Based on these observations, it was suggested that the use of a phosphate binder in which part of the calcium is replaced by magnesium might reduce vascular calcification by, on one hand, reducing phosphate and calcium load and, on the other hand, favoring the beneficial inhibitory effect of magnesium.

However, until today, the effect of CaMg on development and/or progression of vascular calcification has not been investigated. In this experimental study using a rat model with adenine-induced CRF, the effect of CaMg on the development of vascular calcification was investigated and compared with sevelamer carbonate, which has already been proven to exert a beneficial effect on vascular calcification in both experimental and clinical studies.

Results show that hyperphosphatemia was effectively controlled by treatment with 375 and 750 mg/kg of CaMg in adenine-induced CRF rats, doses that are significantly lower than those previously reported in similar experimental studies using other phosphate binders. According to the...
relative phosphate-binding coefficient of each phosphate binder (based on our own unpublished data and those reported in the studies of de Francisco et al.\textsuperscript{11} and Daugirdas et al.\textsuperscript{28}), a dose of 375 mg/kg/day CaMg approximately corresponds to a dose of 750 mg/kg/day of sevelamer. Despite the phosphate-lowering capacity of CaMg and sevelamer, no reduction of serum iFGF-23 levels was observed, which can probably be explained by the severity of the renal impairment inherent to the adenine model and the concomitantly dramatically increased iFGF-23 levels going along herewith. Another explanation could be that the therapeutic period in this animal study was too short to have an effect on iFGF-23.

There is increasing evidence (\textit{in vitro} and \textit{in vivo}) for elevated phosphate to play a causal role in the induction of vascular calcification in the aorta in synergy with calcium,\textsuperscript{29} as it may trigger the transdifferentiation of vascular smooth muscle cells into osteochondrocyte-like cells. The efficient control of serum phosphate by treatment with CaMg resulted in significant reductions of aorta calcifications as measured by the total tissue calcium content and by histology, even at the lowest dose of CaMg. Significantly reduced aortic calcifications in the aorta after treatment with sevelamer were only seen after histological analysis of von Kossa–stained sections of the aorta. Treatment with sevelamer resulted in significantly less calcification in the carotid artery, whereas this was not the case for CaMg. This may imply that the mechanism inducing calcification in the aorta to a certain extent differs from that in the smaller arteries that slightly differ in constitution with respect to, for example, the amount of elastin, collagen, or smooth muscle cells. The calcification process in the peripheral arteries may be triggered by other factors than those defined in more proximal arteries and possibly depends to a lesser extent on circulating serum phosphate concentrations. In this context, it is worth mentioning that sevelamer, apart from its phosphate-lowering capacity, also reduces low-density lipoprotein concentrations and exerts anti-inflammatory effects,\textsuperscript{30,31} aspects that may play a causal role in the calcification process of arteries as well. However, for the femoral artery, we could not observe an effect of any of the phosphate binders on calcification. Additional studies are required to further present evidence for these observations.

### Table 2 | Calcium and magnesium content in the aorta, carotid and femoral arteries

|                | Aorta      | Carotid artery | Femoral artery |
|----------------|------------|----------------|----------------|
|                | Ca (mg/g)  | Mg (mg/g)      | Mg/Ca ratio    |
| Vehicle (n = 14) | 54.5 ± 29.7 | 2.75 ± 1.66   | 0.072 ± 0.064  |
| CaMg750 (n = 14) | 24.9 ± 16.5* | 2.72 ± 1.61 | 0.137 ± 0.065* |
| CaMg375 (n = 14) | 17.8 ± 13.2* | 1.84 ± 1.44 | 0.135 ± 0.074* |
| Sev750 (n = 14)  | 33.4 ± 34.2 | 1.98 ± 1.81   | 0.128 ± 0.103  |

Abbreviations: CaMg, calcium acetate/magnesium carbonate; CaMg375, CaMg 375 mg/kg; CaMg750, CaMg 750 mg/kg; Sev, sevelamer; Sev750, sevelamer carbonate 750 mg/kg.

*Significant difference versus vehicle.

Data are expressed as mean ± s.d.

Tissue calcium, magnesium concentration, and Mg/Ca ratio in the different blood vessels.

### Figure 4 | Calcium X-ray fluorescence and X-ray diffraction patterns of the deposited mineral in the calcified aorta of a uremic rat treated with 375 mg/kg calcium acetate/magnesium carbonate (CaMg). All groups showed similar diffraction patterns.
In addition to its phosphate-lowering capacity, magnesium may also directly influence the development of medial calcification by other mechanisms (Montes de Oca Gonzales et al., abstract FR-PO1195 presented at ASN 2011). It is already known that at low concentrations, magnesium ions can interact with the initiation and growth of calcium phosphate crystals. In this study, microsynchrotron-based X-ray diffraction analysis revealed that the mineral deposited in the calcified regions had the physicochemical characteristics of hydroxyapatite and that there was no evidence for the presence of whitlockite, neither was there any particular difference in the precipitation phase between CaMg, sevelamer, and the non-treated CRF groups. With regard to the absence of whitlockite in this study, it should be mentioned that the Mg/Ca ratios in the aorta of CaMg-treated animals, even though being slightly elevated compared with vehicle, were distinctly lower than the values reported in the study of Verberckmoes et al. using CRF rats with calcitriol-induced vascular calcification.

The inhibitory effect of CaMg and sevelamer on the development of aortic calcification was not reflected by a decreased expression of osteochondrogenic markers. It is remarkable that the expression of sox9, bmp-2, and mmp, which are upregulated in uremic rats, is associated with the presence or absence ('on/off' situation) of aortic calcification. The most probable explanation for the relatively high expression of the osteochondrogenic proteins in the phosphate binder groups is that osteochondrogenic conversion of

Figure 5 | mRNA expression of the different target genes relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the aorta of rats with chronic renal failure (CRF) compared with rats with normal renal function (NRF). The number of animals for each study group is indicated in the bars of Figure 1. Data are expressed as mean ± s.d. bmp-2, bone morphogenic protein 2; CaMg, calcium acetate/magnesium carbonate; CaMg375, CaMg 375 mg/kg; CaMg750, CaMg 750 mg/kg; cbfa-1 core binding factor-α1; mmp, matrix gla protein; Osx, osterix; Sev, sevelamer; Sev750, sevelamer carbonate 750 mg/kg; sox9, sex determining region Y-box 9; TRPM7, transient receptor potential cation channel, subfamily M, member 7; VC, vascular calcification; VK, von Kossa positivity. *Significant difference versus NRF; **significant difference versus VC of the same group.
vascular cells precedes the calcification process. As most of the animals of the CaMg and sevelamer groups still present calcification in the aorta—although it might merely be a mild degree of calcification—one may reasonably accept that the expression of the osteochondrogenic markers was not inhibited. The mRNA expression of the magnesium transporter TRPM7 in the aorta had not changed despite increased serum magnesium levels in the CaMg groups. This is in accordance with recent in vitro studies investigating the effects of magnesium on the osteogenic transdifferentiation process of vascular smooth muscle cells. An inhibitory effect of magnesium on calcium deposition was observed in vascular smooth muscle cells cultured in high phosphate medium to induce calcification. Despite no regulation on transcriptional levels of TRPM7, addition of magnesium to the calcification medium restored or even increased the activity of TRPM7 and prevented calcification, indicating a possible role of this transporter and of magnesium in the transdifferentiation process of vascular smooth muscle cells. Indeed, addition of magnesium inhibited the upregulation of calcification-associated genes like sox9 and msx2 induced by high phosphate in vascular smooth muscle cells.

Treatment with phosphate binders did not result in increased ionized calcium levels in the serum, not even in CRF rats treated with CaMg, which is in line with data from patients treated with this phosphate binder. This is an important finding as free serum calcium rather than protein-bound calcium triggers the development of vascular calcifications. It is worth mentioning that the absence of any increase in serum ionized calcium levels in the CaMg groups versus the vehicle or sevelamer groups was seen despite a tendency toward higher total serum calcium levels. Most probably, this may be explained by the somewhat higher serum pH and bicarbonate (HCO₃⁻) levels favoring the protein binding of calcium (and of magnesium) in the CaMg groups.

Treatment of CRF rats with CaMg750 and CaMg375 dose-dependently increased the serum PTH levels inherent to renal failure. Both doses of CaMg as well as 750 mg/kg sevelamer were overall comparably effective in preventing the development of aortic calcification, quantified by calcium content. Therefore, phosphate binders in which part of the calcium is replaced by magnesium might be a good alternative for calcium acetate/carbonate in dialysis patients with vascular calcification. The beneficial effect of treatment with CaMg on vascular calcification found in this study is promising but requires confirmation in further clinical studies.

MATERIALS AND METHODS

Animal procedures

All animal procedures were approved by the ethical committee of the University of Antwerp. Fifty-six male Wistar rats (ca. 250 g, Charles River, Lille, France) were randomly assigned to four groups: (1) Vehicle (control group), (2) CaMg 750 mg/kg (CaMg750), (3) CaMg 375 mg/kg (CaMg375), and (4) sevelamer carbonate 750 mg/kg (Sev750) (Figure 1). CRF was induced in all groups by feeding a 0.75% adenine/2.5% protein diet for 4 weeks. Before CRF induction, rats were preconditioned on a 1.03% phosphorus diet for 2 weeks. This diet was also given to the animals from week 4 until the time of killing at week 6. All diets were provided by SNNIFF Spezialdiäten (Soest, Germany).

One tablet of the magnesium-based phosphate binder CaMg contains 435 mg calcium acetate, equivalent to 110 mg elemental calcium, and 235 mg heavy magnesium carbonate, equivalent to 60 mg elemental magnesium. The dosage treatments represent the amount of active salt either as MgCO₃ and CaAc in the CaMg ratio or as sevelamer carbonate. At 1 week after initiating the adenine-low-protein diet, treatment with phosphate binders was started by daily oral gavage (7 days/week) until the day of killing (Figure 1). A constant dose volume of 10 ml/kg was respected. Hereto, CaMg was dissolved in 1% carboxymethylcellulose. In order to obtain a manageable gavage solution, sevelamer was dissolved in water instead of 1% carboxymethylcellulose. Control animals (vehicle) were gavaged with 1% carboxymethylcellulose in the absence of phosphate-binding agents.

Animals were housed in metabolic cages during the study period for collection of urine samples (Figure 1). The housing was restricted to 4 h and was followed by blood sampling. Hereto, animals of each group were divided into two subgroups in which urine and blood sampling were performed alternately.

After 6 weeks of CRF, animals were killed by exsanguination through the retro-orbital plexus after anesthesia with intraperitoneal injection of 60 mg/kg pentobarbital (Nembutal, Ceva Santé Animale, Libourne, France).

Biochemistry

Serum calcium, phosphorus, and creatinine were measured on a Vitros 5.1 Fusion auto-analyzer system (Ortho Clinical Diagnostics, Rochester, NY). Serum ionized calcium, pH, and bicarbonate (HCO₃⁻) were measured with iSTAT (Abbott Diagnostics, Wavre, Belgium). Serum magnesium and urinary calcium were measured with flame atomic absorption spectrometry (Perkin-Elmer, Wellesey, MA) after appropriate dilution in 0.1% La(NO₃)₃. Serum PTH was determined at weeks 0, 4, and 6 with the rat PTH IRMA kit (Immunotopics, San Clemente, CA). Measurement of intact FGF-23 was done by ELISA (Kainos Laboratories, Tokyo, Japan). Urinary phosphate was measured using the EcolineS Phosphate kit.
(DiaSys, Holzheim, Germany). Urinary parameters were expressed as mg/g creatinine.

**Evaluation of vascular calcification**

After isolation of the thoracic aorta, tissue was fixed in neutral buffered formalin and cut in ~20 cross-sections of 2-3 mm. These sections were embedded upright in a paraffin block and a 4-µm coupe was stained for vascular calcification with von Kossa's method and counterstained with hematoxylin and eosin. The percentage of calcified area was calculated using Axiovision Release 4.5 image analysis software (Carl Zeiss, Oberkochen, Germany) in which two color separation thresholds measure the total tissue area and the von Kossa-positive area. After summing both absolute areas, the percentage of calcified area was then calculated as the ratio of the von Kossa-positive area versus the total tissue area.

The proximal part of the abdominal aorta and the left femoral and carotid arteries were isolated and weighted on a precision balance. Subsequently, samples were digested in 65% HNO₃ at 60 °C overnight. The calcium and magnesium content of the tissue was measured with flame atomic absorption spectrometry (Perkin-Elmer) and expressed as mg calcium or magnesium per g wet tissue.

Unstained 10 µm-thick aorta sections were used for synchrotron X-ray μ-fluorescence and X-ray μ-diffraction analysis on beamline ID18 of the European Synchrotron Radiation Facility, applying the experimental setup as previously reported by Verberckmoes et al. In brief, a 2-dim 135 mm MAR charge-coupled device-based diffraction camera was used to capture the diffraction patterns. The μ-focused monochromatic X-ray beam with energy of 14.4 keV was used to scan either the synthetic control samples or the aorta samples. By applying this energy, the experiment benefited from the revolver undulator of beamline ID18 that was optimized at this energy. X-ray fluorescence mapping for calcium allowed to identify calcified regions within the aorta sections. Per sample, several line scans of adequate length were recorded to obtain sufficient information and to analyze the spatial heterogeneity of the mineral phases. Synthetic hydroxyapatite (Ca₁₀(OH)₂(PO₄)₁₂), whitlockite (Ca₃(Mg,Fe)₅(PO₄)₃(OH))(PO₄)₂), and brushite (CaHPO₄·2H₂O), prepared following standardized methods, were used as positive controls. These standards were available as powders. The μ-diffraction patterns were analyzed with the X-ray diffraction software package Fit2D.⁴⁰ (European Synchrotron Radiation Facility, Grenoble, France) and after background correction of the integrated X-ray diffraction spectra, the diffraction spectra of the samples were compared with the reference diffraction spectra of the standards from the diffraction database PCPDFWIN version 2.1 (International Centre of Diffraction Data, Newtown Square, PA).

**Gene expression analysis**

Total mRNA was extracted from the distal abdominal aorta with the RNeasy Fibrous Tissue mini kit (Qiagen, Hilden, Germany) and transcribed to cDNA with the High capacity cDNA archive kit (Applied Biosystems, Foster City, CA). The PCR to quantify mRNA was based on the Taqman fluorescence method (ABI prism 7000 sequence detection system, Applied Biosystems). Taqman probes and primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Rn00180266_m1), citrate synthase (cbsa-1 (Rn01512231_m1), sso9 (Rn01751069_m1), mmp (Rn00534632_m1), bmp-2 (Rn14847363_m1), ssox (Rn2769744_s1), and TRPM7 (Rn00586779_m1) were purchased from Applied Biosystems as a Taqman gene expression assay. Each gene was analyzed in triplicate and the expression was normalized to the housekeeping gene GAPDH. Calculations were made to conform with the comparative CT method using aortas of rats with normal renal function as calibrator samples (historical data with a low-protein diet from previous studies performed in our lab), which was given a gene of interest/GAPDH expression ratio of 1.

**Statistics**

Results are expressed as mean ± s.d. unless otherwise indicated. Nonparametric statistical analyses were performed with SPSS 18.0 software (Brussels, Belgium). Statistical differences were investigated with Kruskall-Wallis test followed by Mann-Whitney U-test. Bonferroni correction was applied when more than two groups were compared. P < 0.05 was considered significant.

**DISCLOSURE**

ME Peter, S Steppan, and K Gwadial are current employees of Fresenius Medical Care Deutschland GmbH.

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