Fetuin-A (AHSG) prevents extraosseous calcification induced by uraemia and phosphate challenge in mice

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Abstract

Background. Chronic kidney disease (CKD) is associated with vascular and tissue calcification. The extent of vascular calcification has been identified as an independent risk factor of cardiovascular death in patients on haemodialysis.

Methods. We studied the role of fetuin-A in CKD-associated calcification using a mouse model of graded renal insufficiency generated by nephrectomy and high phosphate diet. We used wild-type and fetuin-A-deficient mice on the calcification resistant genetic background C57BL/6 to study the influence on calcification of CKD, dietary phosphate and fetuin deficiency. Hyperphosphataemia, elevated BUN, hyperparathyroidism and von Kossa histochemistry served as indicators of calcification disease. The expression of osteopontin, a marker of osteoblast-like cell differentiation was analyzed by realtime PCR and immunohistochemistry.

Results. We detected tissue and genotype-specific susceptibility for calcification. Fetuin-A-deficient mice with CKD and high phosphate diet had only a moderately elevated serum calcium phosphate product (6.9 ± 1.4 mmol²/l²), but suffered severe calcification of kidney, heart and lung. In contrast, wild-type mice under the same conditions developed renal calcinosis only despite an elevated serum calcium phosphate product (9.6 ± 0.9 mmol²/l²). Calcification was preceded by the local induction of osteopontin, a marker for osteoblast-like cell differentiation.

Conclusion. Fetuin-A deficiency, CKD and high phosphate diet act synergistically in the pathogenesis of extraosseous calcification.

Keywords: calcification; calcium; chronic kidney disease; osteopontin; phosphate

Introduction

Cardiovascular disease and mortality are dramatically increased in patients with chronic kidney disease (CKD) and account for premature death in 50% of dialysis patients [1]. The extent of vascular calcification has been identified as an independent risk factor of cardiovascular death in patients on haemodialysis [2,3].

It is now generally believed that pathological calcification is driven by an elevated serum calcium phosphate product (the 'passive' or chemical view) as well as by transformation of vascular or mesenchymal cells into osteoblast-like cells becoming mineralization competent (the 'active' or biological view). A vicious cycle may ensue because high extracellular calcium and phosphate favour the phenotypic transformation into osteoblast-like calcifying cells. What triggers the increased calcification associated with renal failure?

Hyperphosphataemia was recognized as an independent risk factor for cardiovascular disease in registry studies [4,5]. It was believed that the mineral homeostasis, which is disturbed in CKD, results in an elevated serum calcium phosphate (Ca-Pi) product sustaining calcium phosphate precipitation. Serum is a metastable solution with respect to calcium phosphate precipitation. Once started, calcification proceeds rapidly, especially in the presence of calcifiable templates like collagen, elastin or cell debris [6]. Therefore, calcification must be actively prevented at all times and in all tissues not meant to calcify [7]. A number of inhibitors of ectopic calcification have been identified using genetically altered mice [8]. Notably, matrix-GLA protein, osteopontin, fetuin-A and pyrophosphate have been shown to interfere with mineralization both in vitro, in cell culture and in vivo. Regardless of the triggering event a chemical precipitation reaction...
concludes the calcification process [9]. Therefore, the efficient inhibition of this final precipitation step is the last-line defence reaction against imminent tissue calcification. Fetuin-A (α2 Heremans Schmid glycoprotein, Ahsg) is a major systemic inhibitor of calcium phosphate precipitation accounting for about 50% of the capacity of serum to inhibit the spontaneous apatite formation from solutions of calcium and phosphate [10]. In adults, fetuin-A is secreted mainly by the liver reaching systemic concentrations of 0.5–1 g/l. An insight to the mechanism of serum fetuin-A inhibition was presented by a study of young rats developing extreme hypercalcaemia 6 h after high-dose etidronate treatment [11]. A high molecular weight complex basically made of fetuin-A, calcium and phosphate was rapidly formed and cleared again from circulation within 24 h. Concomitantly, a large proportion of serum fetuin-A was consumed. This finding indicated that fetuin-A stabilized calcium phosphate as a colloid and mediated its clearing from circulation. This experimental result in rats is in full agreement with our in vitro study on ‘calciprotein particles’, the complex of fetuin-A, calcium and phosphate [10], as well as the recent observation that fetuin-A strongly accumulated in calcified vessel lesions of patients with renal failure [12].

We have previously shown that dialysis and calciphylaxis patients have depressed fetuin-A serum levels accompanied by a reduced capacity of their serum to inhibit Ca-Pi precipitation [13,14]. Low fetuin-A serum levels inversely correlated with CRP as an indicator of inflammation confirming that fetuin-A is a negative acute phase protein [15]. Moreover, fetuin-A deficiency was identified as an inflammation-related predictor of cardiovascular and all-cause mortality, respectively [13].

In this study, we sought to dissect the contribution of fetuin-A, CKD and an elevated Ca-Pi product, respectively, to the development of extraosseous calcification using fetuin-A-deficient mice maintained on the relatively calcification-resistant genetic background C57BL/6. Unlike DBA/2, Ahsg−/− mice, which have a fully penetrating phenotype of extensive soft tissue calcification [14] C57BL/6, Ahsg−/− mice represent ‘borderline calcifying’ mice well-suited to study diet-related metabolic challenges or combined genetic deficiencies, which are typical of multifactorial human disease like atherosclerosis or CKD. We included expression analysis of osteopontin (OPN), an established osteoblast marker in extraosseous calcification [16], as an indicator of disease progression.

**Materials and methods**

**Animals and diets**

The animal study protocol was approved by the local animal welfare committee. We used male mice to avoid gender-dependent variability in renal disease [17]. Fetuin-A-deficient mice (Ahsg−/−) on a C57BL/6 background were generated by 10 successive backcrosses of the original C57BL/6-129/Sv hybrid mice [18] to pure-bred mice obtained from a commercial breeder (Charles River Wiga GmbH, Sulzfeld, Germany). Age-matched 12-week-old wild-type (WT) mice were obtained from the same breeder. Genotyping was performed by PCR and Southern blotting as previously described [14]. Mice were kept in a climate-controlled room (22°C, 45–54% relative humidity) with a 12 h light/12 h dark cycle. Food and water were given ad libitum. The day after completing renal ablation or sham operation, animals received standard low phosphate (LP) chow (Altromin 1324: Altromin GmbH, Lage, Germany) containing 0.9% calcium, 0.7% Pi and 19% protein (mainly soy grist), or high-phosphate (HP) diet (Altromin CI049) containing 0.95% calcium, 1.65% Pi and 17% protein (mainly casein) for the duration of the study. An overview of the study design is given in Figure 1.

**Surgical procedures**

Renal ablation as a model of CKD was achieved by a two-step surgical procedure [19]. Mice 12 weeks of age were anaesthetized by intraperitoneal injection with Avertin (2.5% stock; 15 μl/g body weight). We thermally coagulated the cortex of the left kidney sparing the hilus region through a 2 cm incision of the left abdominal wall. We performed right-sided nephrectomy 2 weeks later. The mice generally recovered well from the renal ablation surgery. Following nephrectomy, 28 out of 87 mice showed signs of severe distress including excessive weight loss, lack of movement, low body temperature and scruffy fur. These animals were euthanized and excluded from the study according to the protocol approved by the animal welfare committee. Most animals were lost in the CKD treatment groups 5–8. According to the initial results, in a second set of experiments, a larger number of animals were enrolled in groups 7 and 8 to attain statistically meaningful results. Hence, the numbers in the different treatment groups do not match, as depicted in Figure 2. Mice were fed standard LP chow or HP diet for 8 weeks. Mice were anaesthetized with isoflurane. Blood was collected by retroorbital bleeding. Mice were killed by cervical dislocation, and kidney, lung, heart and aortic tissues were collected. For histological analysis, the basis of the heart and the thoracic aorta as well as one
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RNA isolation and absolute quantitative RT realtime PCR

RNA was extracted following the RNAlater® and RNeasy® protocols (QIAGEN, Hilden, Germany). Heart and aortic tissues were digested with proteinase K prior to RNA extraction in order to maximize RNA yield. Integrity and amount of RNA were measured by capillary electrophoresis (Agilent Bioanalyzer 2100, Agilent Technologies, Böblingen, Germany). Reverse Transcription and realtime PCR were performed on 100 ng RNA using a commercial RT-PCR kit (Eurogentech, Cologne, Germany) and the ABI 7700 sequence detection system (PE, Applied Biosystems, Inc., Foster City, USA). The PCR reactions were performed in duplicate in 20 μl reaction-mixtures containing 2 μl cDNA from the reverse transcription, 300 nM of each primer and 100 nM of each probe (Eurogentech). OPN-specific primers were derived from EnsEmbl entry ENSMUST0000031243: sense: GCCATGAGATTTGCA GTGATT, antisense: GATCTGGGTGGAGGTGAAGG, probe: FAM-ATGCTCCTCCTCCCTCCCGTG-TAMRA, to yield an amplicon length of 116 bp, spanning an intron. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific primers were derived from EnsEmbl entry ENSMUST0000086934: sense: GGCAATTCAACGCGACAGT, antisense: AGATGGTGATGGGGCTTCCC, probe: FAM-AAG GCCGAGAATGGGAGCTTGTGATC-TAMRA, to yield an amplicon length of 74 bp. Annealing temperatures were 60°C. PCR amplicons were sized using agarose gel electrophoresis (3% low melting agarose) gel electrophoresis for one million copies of GAPDH RNA. Calculations were performed with the Sequence Detection Software (PE, Applied Biosystems, Inc., Foster City, CA, USA).

Histology—Immunohistochemistry

Methyl Carnoy-fixed tissue specimens were embedded in paraffin using an automated tissue processor. Sections were cut in 5 μm thick slices and analysed by immunohistochemical staining for OPN expression. Brieﬂy, sections were dewaxed and rehydrated. Sections were boiled twice for 5 min in buffer (10 mM citric acid, pH 6.0) and rinsed in tap water to retrieve antibodies. Endogenous peroxidase was quenched with 0.03% H2O2 in methanol for 10 min, followed by a brief rinse in PBS and incubation in 4% horse serum (Vector Laboratories, Burlingame, USA) containing the cloned target genes (GAPDH, OPN) and results were expressed as copy numbers of target gene per one million copies of GAPDH RNA. Calculations were performed with the Sequence Detection Software (PE, Applied Biosystems, Inc., Foster City, CA, USA).

Qualitative staining for calcification: von Kossa

Dewaxed and rehydrated sections were imibed in fresh AgNO3-solution (1% in water) for 5 min, rinsed in water (three times for 3 min) and incubated in a solution of 5% Na2CO3 and 9.25% formalin for 1 min. After a second rinse, sections were developed using sodiumthiosulfate (5% in water) for 5 min and counterstained in 0.1% safranin-O followed by a final rinse using tap water. Frozen sections of
mouse embryos containing black staining mineralized bone (embryonic day 18) served as positive controls.

**Quantification of soft tissue calcification**

Calcium was extracted from tissues overnight using 0.6 M HCl. After clearing the extracts by centrifugation (10 000g, 3 min) calcium was determined with a commercial kit employing cresolphthalein complexone chemistry (Randox Laboratories Ltd, Crumlin, GB).

**Radiographic analysis**

Mice were anaesthetized with isoflurane and X-rayed using a Senographe DMR X-ray system (GE Medical Systems, Solingen, Germany) with a magnification of ×1.9 at 25 kV and 35 mA as previously described [14].

**Serum chemistry**

Blood was collected into tubes containing a clotting-aid. Serum was separated by centrifugation at 2000 g and stored at –70 °C until assayed for Ca, P, C-reactive protein and BUN by standard laboratory methods. PTH was determined by a commercial ELISA (Immutopics, San Clemente, CA, USA).

**Statistics**

If not otherwise noted, analysis of variance (ANOVA) with Tukey’s post-hoc analysis was used to test for overall differences in non-size matched experimental groups. Confidence intervals >95% were regarded as significant.

**Results**

**Experimental groups and biochemical data**

*Treatment groups.* We allocated animals to eight groups. Animals were subjected to sham surgery or 5/6 renal ablation. Sham and CKD groups were further subdivided and fed either low phosphate (LP) or high phosphate (HP) chow. Each of the four interventions was performed in WT and Ahsg–/– mice, respectively, resulting in eight different animal groups with a total of 59 animals reaching the end of the study. An experimental outline is given in Figure 1.

*Serum chemistry.* Animals undergoing renal ablation developed CKD indicated by roughly 2-fold elevated BUN serum levels (Figure 2, top panel). Fetuin-A-deficient mice had less pronounced hyperuricaemia than WT animals (group 6 vs 5 and group 8 vs 7). Fetuin-A-deficient mice also displayed a tendency to reduced serum calcium reaching statistical significance on HP diet (group 4 vs 1, P < 0.05) and when CKD was combined with HP (group 8 vs 1, P < 0.001). Serum phosphate increased on HP diet. The increase was small in sham-operated animals (groups 3 vs 1 and 4 vs 2) and attained frank hyperphosphataemia in CKD animals (groups 7 vs 5 and 8 vs 6). Fetuin-A-deficient mice had lower serum phosphate on HP than WT mice (group 4 vs 3). This difference was even greater when HP diet was combined with CKD (group 8 vs 7, P < 0.001). The serum Ca-Pi product mirrored the serum phosphate. Again, fetuin-A-deficient mice displayed lower values than their respective WT counterparts especially in the HP-CKD group (group 8 vs 7, P < 0.05). PTH serum levels of WT and Ahsg–/– sham mice were indistinguishable (group 1: 78 ± 28 ng/ml vs group 2: 68 ± 29 ng/ml). HP diet was associated with hyperparathyroidism in all mice. The combination of HP diet and CKD further increased the PTH serum levels regardless of genotypes (group 7: 879 ± 568 ng/ml; group 8: 731 ± 465 ng/ml; P < 0.05; both groups vs group 1).

Body weight was similar in all experimental groups (Figure 2). In general, there was a tendency to reduced body weight in the CKD mice, which only reached statistical significance comparing group 8 (27 ± 2 g) with group 1 (32 ± 2 g) (group 8 vs 1, P < 0.05). In keeping with the animal welfare protocol, we removed from the study and euthanized all animals showing signs of severe distress. This clearly caused bias towards animals with a higher tolerance of CKD and CKD/HP. The high mortality associated with CKD required extra animals to be entered into the CKD/HP groups to attain statistically significant numbers for the comparison of groups 7 and 8, which differ in fetuin-A genotype. No such increase in animal numbers was attempted in groups 6 and 7. Despite clearly established CKD these animals behaved like non-CKD animals in all parameters regarding calcification including von Kossa histochemistry, serum calcium/phosphate and PTH.

**Soft tissue calcification.** C57BL/6 WT mice displayed no visible signs of soft tissue calcification in myocardium, aorta, lung and kidney judged by von Kossa histochemistry (Figure 3A, C, E and G). In contrast, fetuin-A-deficient mice had sporadic soft tissue calcification detected by von Kossa histochemistry but not by radiology (data not shown). Calcification in WT mice on CKD and HP diet (group 7) was restricted to kidney tubules (data not shown). The mild calcification phenotypes of groups 1 through 6 and the nephrocalcinosis in group 7 were strongly exacerbated in Ahsg–/– mice on CKD and HP diet (group 8). These mice suffered calcification in myocardium, lung, heart valve and kidney tissue (Figure 3B, F, H and J) but not in aorta (Figure 3D). All other experimental groups did not show overt signs of calcification with the exception of heart valves. Heart valves had calcified lesions ranging from about 20% in WT mice (group 1) to over 80% of Ahsg–/– mice on CKD and HP (Figure 3I and J) indicating that heart valves are very prone to calcification even in the relatively calcification-resistant genetic strain C57BL/6.

We quantified the extent of soft tissue calcification by chemical analysis of tissue calcium (Figure 4). Kidney, myocardium and lung from sham-operated
Fig. 3. Extent and localization of soft tissue calcification. Representative photomicrographs of von Kossa-stained sections from non-calcifying experimental group 1 (A, C, E, G, I) and the Ahsg-deficient, CKD and HP animals of experimental group 8 (B, D, F, H, J) in different organs: myocardium (A, B), aorta (C, D), lung (E, F) kidney (G, H) and aortic valve leaflets (I, J).
WT mice on LP diet contained between 1.3 mg and 2.2 mg calcium per gram tissue. Similar calcium contents were measured in tissues of all sham-operated mice and in CKD mice on LP diet. In contrast, nephrectomized Ahsg−/− mice on HP diet had 15-fold increased calcium in their kidneys (group 8 vs 1, \( P < 0.05 \)), 5-fold in myocardium (group 8 vs 1, \( P = \text{n.s.} \) by ANOVA testing; \( P = 0.027 \) by Mann–Whitney testing) and 5-fold in lungs (group 8 vs 1, \( P < 0.05 \)), confirming the histology presented in Figure 3 by analytical chemistry. Please note that in WT mice on CKD and HP there was a significant increase in kidney calcium (20-fold group 7 and group 8 vs 1, \( P < 0.05 \)) suggesting that fetuin-A plays a minor role in the prevention of nephrocalcinosis. Taken together, our data confirm the importance of fetuin-A as a systemic inhibitor of soft tissue calcification. We conclude that a rank order of proneness to calcification exists among organs, with the kidney being most vulnerable followed by myocardium and lung. The unexpected high incidence of heart valve calcification clearly merits further study to develop the CKD-HP model into a model of human heart valve calcification.

**Osteopontin mRNA induction.** Osteopontin is a marker associated with osteoblast-like cells and macrophages. It has been postulated that osteopontin is expressed before overt calcification develops and both, indirect and direct roles of osteopontin in calcification inhibition have been demonstrated. In particular, OPN was able to reverse calcification in an implant model [20]. Against this background, we determined OPN expression using quantitative RT-PCR (Figure 5). WT sham mice on LP diet (group 1) expressed low levels of OPN mRNA in aorta, myocardium, kidney and lung tissue (3, 2, 85 and 2000 copies OPN, respectively, per one million copies GAPDH). HP diet and fetuin-A deficiency were associated with slightly elevated OPN expression. HP diet induced a significant rise in OPN mRNA induction in kidneys of both genotypes (\( P < 0.05 \); groups 3 and 4 vs 1). Treatment groups 7 and 8, which suffered the most renal calcification, had an even higher OPN induction, 15-fold and 25-fold, respectively, compared to untreated WT mice (\( P < 0.01 \); groups 7 and 8 vs 1). In summary, OPN mRNA was up-regulated in all tissues of fetuin-A-deficient mice suffering CKD and HP diet. Notably, strong up-regulation was detected in the aorta of these mice despite the clear absence of calcification in von Kossa histology. Similarly, we detected a strong OPN induction in lung tissue of WT mice suffering CKD and HP (group 7) without overt calcification. These results indicate that OPN expression closely mirrored and even preceded calcification indicating that OPN may serve as an early marker of imminent calcification.

We confirmed the mRNA results on the protein level by immunohistochemistry. OPN protein was undetectable in non-calcified myocardium and aorta (Figure 6A and C). Low basal OPN protein staining was detected in non-calcified lung along the airway epithelia and in a few positively-staining alveolar cells and monocytes within the pulmonary capillaries (Figure 6E). In the kidney, OPN antibody stained positive in non-calcified tubular epithelia sparing the glomeruli (Figure 6G). In calcified tissues, OPN staining was strongly enhanced (Figure 6B, D, F and H). In lung (Figure 6F) and kidney (Figure 6H), OPN immunoreactivity co-localized with calcified lesions. We failed to detect OPN in the aorta wall, which also never calcified as stated above. The diffuse OPN staining localized to the adventitia of the aorta in CKD Ahsg−/− animals (Figure 6D) and corresponded to high-level mRNA expression in excised aortas. This suggested that the majority of OPN mRNA was actually derived from adventitia, not from aortic wall. In summary, OPN expression positively
correlated with calcification risk (Figure 6B, F and H) rendering this protein a marker of imminent as well as actual calcification.

**Discussion**

We studied the role of fetuin-A in the development of extrasosseous calcification in a mouse CKD model. Human studies in haemodialysis patients are complicated by a complex CKD aetiology and the multiple co-morbidities precluding a detailed analysis of the relative contribution of suspected activating and inhibitory mechanisms of calcification and their sequence of action. To this end we studied the influence of fetuin-A deficiency, high phosphate diet and CKD on calcification using C57Bl/6 WT mice and age- and sex-matched fetuin-A-deficient ‘borderline calcifying’ C57Bl/6, Ahsg−/− mice.
Increased BUN showed that CKD was readily induced in both WT and fetuin-A-deficient mice following 5/6 nephrectomy. Additional dietary phosphate (HP) led to significant hyperphosphataemia, especially in CKD mice. Serum PTH was elevated in all mice on HP diet to reduce intestinal phosphate absorption and renal reabsorption by down-regulating sodium-phosphate cotransporters of the SLC34 family [21]. Using the graded disease model presented here, we could reproduce key metabolic derangements of CKD. This model revealed profound tissue-specific differences in calcification susceptibility indicating a variable contribution of activating and inhibitory principles depending on the tissue type.

Previous studies in rodent CKD models [22–24] and fetuin-deficient mice [14,25] highlighted the contribution of CKD, phosphate load and fetuin-A deficiency in the pathogenesis of soft tissue calcification. Here we show that hyperphosphataemia, CKD and fetuin-deficiency act independently and additively in that the calcification was most severe when all three risk factors were combined. CKD combined with phosphate overload was necessary and sufficient to cause nephrocalcinosis and kidney parenchymal calcification regardless of the fetuin-A genotype suggesting that fetuin-A plays a minor, if any, role in the aetiology of nephrocalcinosis. This point needs, however, further study in the light of clear renal tubular fetuin-A expression both on the protein and on the mRNA level [26]. A recent report stated that exosomal fetuin-A is a novel urinary biomarker of acute kidney injury [27]. In addition, a significant negative correlation of serum fetuin-A with free phosphate levels was recently reported indicating that fetuin-A is a determinant of serum phosphate [28]. This finding is compatible with a role of fetuin-A as a (calcium)phosphate carrier in intestinal uptake, renal secretion, blood transport or both. The transported entity may well be the calcioprotein particles or the fetuin-A/calcium phosphate complex that we and others have described in great biochemical detail [10,29,30]. Taken together, these findings raise the possibility that renal tubular fetuin-A may constitute a local protective mechanism operating at a known predilection site of calcification. In this hypothetical scenario, tubular fetuin-A could prevent renal calcification in a fashion recently shown in matrix vesicle and apoptotic vesicle-mediated calcification of smooth muscle cells [31]. This fetuin-A-mediated inhibition mechanism is, however, obviously overwhelmed in CKD and especially in CKD/HP.

Arterial calcification, a hallmark of CKD-associated vascular disease [32], was conspicuously absent in our animals. Considering the time vessel calcification requires to develop in humans, the observation period in this study may have been too short. In addition, the vasculature of our mice was healthy and undamaged at the onset of the experiment in contrast to vessels of humans with CKD, characterized by a high prevalence of known risk factors for vascular damage and endothelial dysfunction (hypertension, dyslipidaemia, etc.). In addition, we showed that Ahsg−/− mice up-regulated OPN, which may have compensated for the fetuin-A deficiency. This is supported by the finding that aortic valves, which lack VSMC and hence OPN [33] calcified in about 20% of WT mice and in 80% of CKD, HP Ahsg−/− mice. An association of reduced fetuin-A serum levels and aortic valve calcification was recently also determined in CAPD patients [34].

One clinically salient finding of our study is that calcification in fetuin-A-deficient CKD mice on HP diet proceeded at a serum Ca-Pi product close to the normal range. Vice versa, the presence of fetuin-A increased the tolerance of even highly increased Ca-Pi serum levels in WT mice. Ex vivo, fetuin-A prevents Ca-Pi precipitation by forming soluble colloidal spheres containing basic calcium phosphate [10,35]. Our data support the view that fetuin-A is an efficient solubilizing agent of Ca-Pi and thereby inhibits calcium phosphate deposition also in vivo. We hypothesize that fetuin-A-deficient mice were unable to develop pronounced hyperphosphataemia because precipitation occurred before the serum Ca-Pi product rose significantly. In line with this hypothesis, fetuin-A-deficient CKD mice on HP diet (group 8) displayed hypocalcaemia, not hypercalcaemia, despite the observed excessive tissue calcification. Untreated fetuin-A-deficient mice already tended to have lower serum calcium than WT mice (group 2 vs group 1). This difference attained statistical significance on HP diet (group 1 vs group 4) and was most pronounced when HP diet and CKD were combined (group 1 vs group 8). Interestingly, similar situations have been reported in humans in that profound calcification was associated with hypocalcaemia and hypophosphataemia even if the calcification process was triggered by an excess of phosphate ingestion with a transient rise in serum phosphate [36].

Vascular smooth muscle cells up-regulate osteoblastic genes including OPN in response to high Pi levels [37] and express OPN in calcified arterial lesions [38,39]. Our study confirms a close association between the degree of calcification and the extent of OPN expression corroborating the hypothesis that OPN expression increases with calcification risk. Regardless of the individual factor increasing calcification risk (fetuin-A deficiency, hyperphosphataemia or CKD), OPN mRNA was elevated in the kidney, lung and aorta already before clear histological evidence of calcification. Therefore, OPN up-regulation may be regarded as a danger response to imminent calcification.

We conclude that the serum Ca-Pi product is a critical but not the sole determinant for the onset of pathological calcification in CKD. In fact, following
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A phosphate-rich diet for 8 weeks, fetuin-A-deficient CKD mice displayed an almost normal serum Ca-Pi product, but nevertheless strongly calcified while the WT mice were still protected at even more elevated and potentially hazardous serum ion concentrations [40]. We suggest, including in clinical studies on extraneous calcification in HD patients, a precipitation test which measures the overall capacity of serum to inhibit Ca-Pi precipitation and the measurement of fetuin-A serum levels.

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Conflict of interest statement. None declared.

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