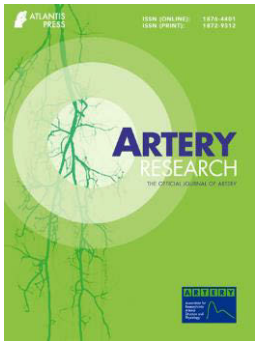

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Uraemic vascular damage and calcification in children on dialysis

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Introduction

Cardiovascular disease (CVD) is the most common cause of death in patients with chronic kidney disease (CKD).[1] Structural and functional vascular abnormalities and arterial calcification begin early in the course of renal decline and can be found even in children, contributing to their high mortality risk.[2,3] For many years vascular calcification was thought to be a mere physicochemical 'dumping' of calcium (Ca)–phosphate (P) in dead or dying cells. However, in recent years, converging evidence from *in vitro* studies, molecular genetic techniques and human single-gene defects have shown that vascular calcification involves a complex interplay between promoters and inhibitors of calcification.[4] Indeed, calcification in the vasculature has many similarities to bone formation and is now regarded as an actively regulated process. The uraemic milieu provides a 'perfect storm' for accelerated calcification: mineral dysregulation, vascular smooth muscle cell (VSMC) damage, perturbation of circulating and cellular inhibitors of Ca and P precipitation, inflammatory insults and co-existing pro-atherosclerotic risk factors all converge in advanced CKD.

This review discusses our current understanding of the process of vascular calcification focusing specifically on Ca and P mediated vascular damage, and linking clinical and basic research experimental findings by using a novel model of intact human arteries in culture.

Epidemiology of cardiovascular disease in paediatric CKD patients

Children with CKD, particularly those on dialysis, have a significant burden of cardiovascular disease, although this is often clinically silent. The United States Renal Data Systems (USRDS) report that 23% of all deaths among patients who received renal replacement therapy (RRT) as children were from cardiovascular causes.[2] An independent and graded association between renal function and cardiovascular events and death has been shown, stressing the importance of recognising and controlling modifiable risk factors from the earliest stages of CKD.[5]

Risk factors for the development of cardiovascular disease

CKD patients have a higher prevalence of both the 'traditional' Framingham risk factors (such as dyslipidemia,

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hypertension, diabetes, smoking and physical inactivity) as well as non-traditional risk factors that are both disease-related (e.g. dysregulated mineral metabolism, anaemia, hypertension and fluid overload, inflammation and oxidative stress) and potentially treatment-related (e.g. calcium overload from dialysate and calcium-based phosphate binders and vitamin D therapy). A number of epidemiological and clinical studies have implied that dysregulated mineral metabolism is largely responsible for the typical arterial medial calcification of CKD.[6]

Block et al. first reported that elevated P levels are an independent risk factor for increased mortality in adult dialysis patients.[7] Large observational studies have also correlated serum Ca levels with increased mortality in hemodialysis patients and have shown that the greatest mortality risk is seen when high Ca and P levels co-exist.[8] Using surrogate measures of vascular injury, a number of studies in children with CKD have shown that mineral dysregulation leads to VSMC damage and calcification.[3,9–13]

Surrogate measures of cardiovascular disease in CKD

A number of surrogate measures of cardiovascular changes are widely used in research studies. These include high-resolution ultrasound to measure carotid artery intima media thickness (cIMT) indicating structural changes in the arterial tree, carotid distensibility and aortic and brachio-radial pulse wave velocity (PWV) to measure stiffness or loss of distensibility, echocardiography (ECHO) to measure the presence, type and degree of left ventricular hypertrophy (LVH), and multi-slice CT scan to demonstrate direct evidence of calcification in the coronary arteries, cardiac valves and aortic root. These surrogate measures are validated and correlate well with cardiac-related events, but their sensitivity in detecting early vascular changes is not known. In addition, these tests are unable to differentiate intimal from medial calcification and cannot provide a mechanistic insight into the nature of the vascular injury.

Models to study vascular calcification in CKD

Model systems that have been developed to study vascular damage and calcification include animal knock-out models, VSMC explant cultures and organ cultures of intact vessel rings. Animal knock-out models have provided insights into the effects of single-gene defects and the crucial role of calcification inhibitors, such as fetuin-A[14] and matrix Gla-protein (MGP),[15] in preventing ectopic calcification. Major mechanistic insights into the process of vascular calcification have come from *in vitro* studies, in particular, studies utilizing human VSMCs in culture.

In vitro studies using human VSMCs have shown that vascular calcification is a highly regulated process with many similarities to bone formation.[16] A mineral imbalance can induce VSMC apoptosis[17] as well as cellular adaptation and vesicle release with these small membrane-bound bodies forming a nidus for the deposition of basic Ca–P in the form of hydroxyapatite.[18] In the healthy vessel wall, vesicles are loaded with physiological inhibitors of calcification such as fetuin-A[19] and MGP[20] that limit their mineralization potential, but evidence suggests that these proteins may be deficient or nonfunctional in patients with CKD. Also, as part of the mineralization process, VSMCs change to an osteo/chondrocytic phenotype that is characterized by the upregulation of bone-specific transcription factors and matrix proteins, including Runx2/Cbfa1 and alkaline phosphatase.[21]

However, explanted VSMCs lack the matrix and architecture of a normal vessel and undergo substantial phenotypic changes so that they may no longer be representative of contractile VSMCs.[22] In contrast, vessel rings have an intact matrix structure including elastic lamellae, the initial site of calcification in the vessel wall, and VSMC can maintain a normal contractile phenotype for a prolonged period. In addition, vessels derived from CKD patients can be experimentally manipulated *in vitro* to study the effects of mineral dysregulation or of other potential uraemic toxins on the vasculature. Moreover, clinico-pathological correlations can be derived by comparing *ex vivo* findings in the vessels with the patients' clinical and biochemical parameters and clinical measures of cardiovascular damage or calcification.

We have developed a model of intact human arteries from children with CKD (pre-dialysis and dialysis patients) and age-matched healthy subjects, to describe the natural history of calcification *in vivo*,[23] and then exposed vessel rings from these to high Ca and P levels to mimic uraemic conditions. We used medium-sized muscular arteries that are routinely removed at omentectomy during a peritoneal dialysis catheter insertion or at renal transplantation and compared these with mesenteric arteries removed at planned intra-abdominal surgery in disease-free, age-matched controls. Children provide a good opportunity to study uraemic influences on the arterial wall because they have fewer pro-atherosclerotic risk factors, which are major confounders in similar adult studies.

In vivo changes in vessels from pre-dialysis and dialysis patients[23]

We showed quantitative evidence that Ca accumulation in the vessel wall begins pre-dialysis, and that accelerated calcification in dialysis vessels is likely triggered not just by a raised Ca and P levels, but by factors specific to the dialysis milieu. In dialysis but not the pre-dialysis or normal vessels, the Ca loading was associated with reduced number of smooth muscle cells as a result of apoptotic cell death. Also, dialysis vessels had undergone osteo/chondrocytic differentiation with an upregulation of bone marker proteins cbfa-1/runx2. There was an incremental increase in the deposition of vesicle proteins annexin VI, MGP, and fetuin-A through pre-dialysis to dialysis, as well as the presence of vesicles and dying VSMCs within dialysis vessels that had not yet developed overt calcification.

We hypothesize that Ca accumulation in the vessel begins in response to increased Ca and P, but protective mechanisms such as adequate mineralization inhibitor levels and extrusion of intracellular Ca via vesicle release

preserve normal VSMC function. In the dialysis milieu, damage-inducing agents that include continued exposure to high, and possibly worsening, Ca and P lead to apoptosis which in turn increases local Ca levels[24] and potentiates osteo/chondocytic differentiation of smooth muscle cells. Also, in normal and pre-dialysis vessels vesicles are loaded with calcification inhibitors, including fetuin-A and MGP that act to limit their calcification potential. The circulating protein fetuin-A is greatly reduced in dialysis and the form of MGP in the calcified dialysis vessels was the unmodified Glu form that has a much reduced capacity to inhibit calcification.[25–27]. With time in the dialysis milieu, vesicle release and VSMC damage increases, resulting in a reduced capacity of the VSMCs to handle Ca-overload and to produce or incorporate inhibitors.

Correlating vessel changes with patients' clinical and biochemical markers and vascular scans[23]

In the above study we correlated the vessel Ca load with the patients' clinical, biochemical and vascular measures (cIMT, PWV, and multi-slice CT scan). The Ca load showed a strong correlation with the patients' serum Ca $\times$ P product in all CKD vessels. The increased Ca load was independent of the patients' age and related only to their time on dialysis. There was no increase in Ca load with increasing time spent in CKD stage IV or V before initiating dialysis, but significantly greater calcification was seen with increasing time on dialysis and was correlated with the induction of apoptosis.

Although Ca loading was evident in both pre-dialysis and dialysis, but evidence for vascular remodeling (increased cIMT and neointima formation) was observed only in dialysis vessels with the highest Ca loads. Potentially, this implies that there may be a causal relationship between Ca loading and increased susceptibility to vessel wall damage and remodeling, however, currently available clinical tools are not sensitive enough to detect what may be functionally significant vascular damage in CKD. This suggests that a normal or negative result of vascular measures must be interpreted with caution.

Mechanistic insights into Ca and P mediated vascular damage — *in vitro* studies[28]

To date studies into the mechanisms that drive calcification in CKD vessels have been hampered by the lack of an appropriate *in vitro* model. To address this, we used vessel rings from intact human arteries used in the above study[23] and tested the response of vessels (from pre-dialysis and dialysis patients and age-matched healthy subjects), to exposure to high Ca and P levels, mimicking uraemic conditions.

Vessels from healthy controls did not calcify even after long-term exposure to supra-physiological levels of Ca and/or P *in vitro*, while pre-dialysis, and to a much greater extent dialysis vessels, calcified. This suggests that normal VSMCs possess intact inhibitory pathways that prevent calcification whereas vessels from pre-dialysis and dialysis patients were susceptible to calcification, due to their prior exposure *in vivo* to the CKD milieu, which damaged the VSMCs and/or compromised their inhibitory mechanisms, thus 'priming' the vessels for calcification.

Consistent with previous work using VSMC explants,[18] this study clearly demonstrated that for a fixed Ca $\times$ P product, elevated Ca was a more potent stimulus to induce calcification than elevated P. Indeed, calcification in CKD vessels was most strongly associated with VSMC death, due to Ca and P induced apoptosis, with calcification ameliorated by treatment with the pan-caspase inhibitor ZVAD. A significant phenotypic adaptation that VSMCs undergo in response to elevated extracellular Ca is vesicle release.[17] Vesicle release is thought to be an adaptive response, as vesicles extrude Ca from the cell, providing protection from intracellular Ca-overload.[29–31] However, this adaptive response promotes ECM calcification if the vesicles are not loaded with calcification inhibitors, such as fetuin-A and MGP that will block mineral nucleation. In this study, annexin VI staining, indicative of vesicle deposition, was increased in CKD vessel rings in response to Ca + P and EM showed that many of the vesicles released in dialysis vessels contained crystalline apatite. Thus, when inhibitory proteins are functional, vesicle release is protective, however when inhibitors are lacking, vesicles become procalcific.[18,23]

Electron microscopy confirmed increased deposition of vesicles containing crystalline Ca and P in the extracellular matrix in dialysis vessels. In contrast, in normal vessel rings, vesicle deposition and calcification did not occur, but instead extensive intracellular mitochondrial damage was observed. This implies that VSMCs in CKD vessels were 'preadapted' to release vesicles due to their prior exposure to dysregulated mineral metabolism *in vivo* and were phenotypically modified when compared to normal contractile VSMCs. The factors that induce VSMCs to become 'vesicle releasing' cells are unknown but maybe linked to VSMC osteogenic differentiation.[21,32] The observation that after long-term exposure to high Ca + P *in vitro*, VSMCs in normal vessels exhibited intracellular Ca-overload and mitochondrial calcification further supports the idea that vesicle release is protective. We hypothesize that these adaptive changes may provide survival benefits in pre-dialysis, but are overwhelmed in dialysis, leading to VSMC apoptosis and accelerated calcification.

Clinical implications of experimental studies[23,28]

These studies, for the first time, provide quantitative evidence that Ca accumulation in the vessel wall begins pre-dialysis and that factors specific to the dialysis milieu trigger accelerated calcification. This suggests that stringent measures to control the serum P levels and also to limit the Ca load to patients (from P binders and dialysate) should be practiced, beginning from the pre-dialysis stages. As elevated Ca in the context of high P is a major inducer of VSMC apoptosis, even transient hypercalcemic episodes, particularly in the already 'stressed' cells of dialysis patients, may promote vascular calcification. While free (ionized) serum Ca levels are tightly regulated, episodic increases, as seen during hemodialysis or with the use of

vitamin D analogs or Ca-based P binders, may potentially influence vascular calcification. Importantly, although Ca accumulation begins pre-dialysis, it progresses extremely rapidly on dialysis, supporting the need to avoid dialysis and perform preemptive renal transplantation wherever possible.

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