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Received for publication: 30.10.2015; Accepted in revised form: 24.1.2016

Nephrol Dial Transplant (2016) 31: 1583–1587
doi: 10.1093/ndt/gfw048
Advance Access publication 27 April 2016

FULL REVIEW

Fetuin-A-containing calciprotein particles in mineral trafficking and vascular disease

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ABSTRACT

Calcium and phosphate combine to form insoluble precipitates in both inorganic and organic materials. This property is useful biologically and has been used by numerous organisms to create hard tissues, a process referred to as biomineralisation [1]. In humans, calcium and phosphate combine to form useful crystal structures largely composed of calcium hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] and these are essential in the growth, maintenance and strength of parts of the skeleton and other structures like teeth. However, it remains unclear how the body achieves the exquisite specificity involved in biomineralisation. In ageing and disease, these pathways are perturbed, resulting in ectopic calcium crystal deposition impairing tissue function and, interestingly, frequently accompanied by simultaneous loss of mineral from sites where it is useful (e.g. bone). One paradigm for this maladaptive situation is renal failure; a situation that we know is associated with vascular stiffening and calcification, along with mineral loss from the skeleton. Mineral trafficking is a loose term used to describe the movements of calcium salts around the body, and new insights into these pathways may explain some of the problems of previous models of bone mineral disease in renal failure and point to potential future therapeutic strategies.

Keywords: calcification, calcium, fetuin, mineral metabolism, phosphataemia

BIOMINERALISATION

The fact that hydroxyapatite crystals do not form spontaneously in the relatively supersaturated serum (based on the activities of calcium and phosphate) is related to the mineral binding roles of circulating proteins (e.g. Fetuin-A) that limit growth of spontaneously forming crystal nidi, thus limiting mineral formation [2]. A number of experiments attest to the damaging effects of pure hydroxyapatite crystals on cells, which cause profound pro-inflammatory changes, but whose effects are

attenuated or abrogated by the presence of such proteins [3]. Thus, mammals have evolved efficient mechanisms to chaperone mineral until it can precipitate safely at the correct mineralization sites (e.g. in the bone). Further, it is also clear that a mineral–serum interface is essential to buffer serum mineral concentrations on a minute-to-minute basis to prevent dangerous excursions in the levels of ionized mineral in the serum [4, 5], while responding to endocrine signals to change this equilibrium in a longer time frame. Balancing these complex and sometimes competing roles is clearly complex, but exciting to explore.

The biomineralisation process is tightly controlled by a number of factors, including a series of proteins that appear to be crucial in directing the assembly of mineralized structures. In humans, the apatite crystals that are required for bone formation start as nanocrystals. While apatite crystals can grow *in situ* (e.g. in bone), nanocrystalisation and calcification nidi may also be formed in extracellular spaces or intracellularly and may be secreted in matrix vesicles or other export packages. At sites of mineralization surfaces, calcification is facilitated by a specialized collagen matrix, rich in glycosylated collagen I and with well-defined spatial configuration [1].

FETUIN-A

One key regulating protein protecting cells from 'naked' hydroxyapatite in mammalian systems is the protein fetuin-A (Fet-A or α_2-Heremans-Schmid glycoprotein) [6]. Fet-A is mainly synthesized in the liver, where it is heavily glycosylated and secreted into the plasma where it circulates at high concentrations [7]. Fet-A has a high affinity for apatite and thus selectively accumulates as one of the major non-collagenous protein components (~25%) in bone and teeth [8–10]. The high affinity of Fet-A for apatite also explains the co-localization of this protein with ectopic mineral deposits found in the vascular wall and other calcified soft tissues [11–14]. Pivotal studies by Jahnen-Dechent *et al.* [15] showed that Fet-A has a particularly high affinity for nascent mineral and inhibits its precipitation from supersaturated solutions in addition to *de novo* apatite formation by mineralizing rat osteoblast cultures. Consistent with this, studies in mice rendered deficient for Fet-A show widespread calcification of the soft tissues and established this rather enigmatic protein as a potent systemic inhibitor of extraosseous mineralization [16–20]. Furthermore, there is *in vitro* and *in vitro* evidence that Fet-A plays a central role in uptake of mineral by vascular smooth muscle cells (VSMC), and free Fet-A may function to limit calcification here by inhibiting caspase cleavage and apoptosis, which are key events in vascular calcification [21]. In humans, Fet-A deficiency has been consistently associated with increased arterial calcification scores and higher mortality rates [22, 23]. In kidney disease, low circulating Fet-A concentrations are associated with progressive aortic stiffening [24] and calcification [25].

CALCIPROTEIN PARTICLES

Fet-A inhibits mineralization by shielding nascent nanocrystals of calcium phosphate from further growth through the formation of an outer protein monolayer or corona—conceptually akin to the manner in which apolipoproteins coat their insoluble lipid cargo [2, 6]. Mutational analysis has demonstrated that mineral stabilization is mediated by an array of regularly spaced acidic residues (Asp or Glu) positioned on an extended β-sheet of the D1 domain in a Fet-A:calcium ratio of ~1:15 [6]. The interaction of pre-formed mineral, Fet-A and, to a lesser extent, other acidic serum proteins (e.g. albumin), generates colloidal (soluble) complexes termed by Jahnen-Dechent *et al.* as calciprotein protein particles (CPP), in analogy to lipoprotein particles that transport insoluble lipid [6].

CPP formation is biphasic, starting with the aggregation of small clusters of fetuin-A-bound mineral ions (calciprotein monomers) to form spherical nanoparticles containing amorphous calcium phosphate called primary CPP (CPP-I). Once in the circulation, these particles may be removed by class A scavenger receptor–mediated pathways expressed in the reticuloendothelial system [26] or undergo rearrangement into more densely packed needle-shaped particles called secondary CPP (CPP-II) that contain mineral in crystalline phase (Figure 1). This transformation process is likely due to nanoparticle growth by accretion of mineral and structural

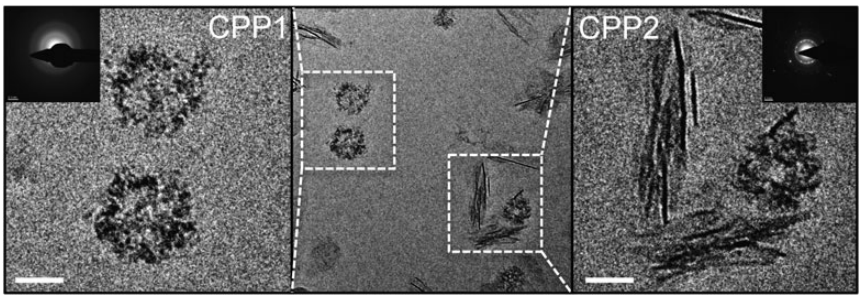

FIGURE 1: Cryo-TEM analysis of fetuin-A-containing CPP isolated from uraemic human serum showing a heterogeneous population of mineral-containing nanoparticles (central panel). CPP were enriched by differential centrifugation and immuno-capture as described previously [3]. Magnified regions show amorphous calcium phosphate–containing CPP-I (left panel), which undergo spontaneous rearrangement to larger needle-shaped CPP-II (right panel) containing mineral in a more densely packed crystalline phase, e.g. octacalcium phosphate/hydroxyapatite (bar = 20 nm). Insets depict typical electron diffraction patterns of each particle 'type'. As described in the text, CPP-I-to-CPP-II transformation is modulated by a number of known promoter and inhibitory factors.

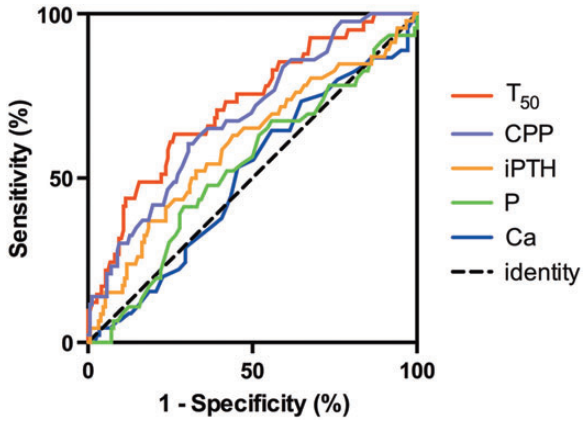

FIGURE 2: Superior discriminative performance of serum T50 and CPP compared with other conventional mineral markers for all-cause mortality in patients with Stage 3 and 4 CKD [31]. Area under the curve (AUC): T_{50} 0.74 (0.65–0.82); CPP 0.69 (95% CI 0.60–0.77); intact PTH (iPTH) 0.60 (0.50–0.70); phosphate 0.53 (0.44–0.63); calcium 0.50 (0.41–0.60). Dashed black line no discriminative ability.

reorganization into a more regular lattice structure with a lower free energy, a process known as 'ripening'. CPP ripening is entirely related to the physicochemical properties of proteins and mineral present, and proceeds spontaneously in a supersaturated solution of calcium and phosphate without the requirement for cellular involvement. A number of factors are known to modulate this transition, including the concentration of Fet-A; albumin; the ionic activity of calcium, phosphate and magnesium; pH; temperature and time [2, 6, 27]

Detection of these particles has to date been mainly by differential centrifugation and ELISA immunoassay for the Fet-A protein [28]. Using such methodology, serum CPP appear undetectable in healthy adults but elevated in patients with CKD, increasing in a stepwise manner with worsening renal function [28]. In the pre-dialysis setting we found that CPP levels were strongly associated with systemic inflammatory markers and aortic stiffness [29]. Others have reported strong correlations between circulating levels and coronary artery calcification scores [28]. Intriguingly, we have also found that serum CPP are elevated in patients with chronic rheumatological disease but normal renal function, providing further evidence of the link between inflammatory and pro-calcific processes [30]. Importantly, measuring the levels of these circulating CPP-II in patients has impressive prognostic significance in CKD [31], being highly predictive of mortality (Figure 2). The ability of a given patient serum to resist the ripening process of these particles *in vitro* in an artificial milieu of supersaturating calcium and phosphate, the so-called calcification propensity (T_{50}) [32], has also been found to have prognostic significance in CKD and transplantation [31, 33], suggesting that as biomarkers they perform well.

Our understanding of CPP metabolism is incomplete, but *in vitro* work has shown that CPP are taken up by various cell types, in particular cells of the monocytic/macrophage lineage, and there is accumulating evidence that CPP-I and CPP-II provoke very different cellular responses, with the latter causing pro-inflammatory cytokine release (especially IL-1β) and reactive oxygen species [3]. The mechanism of this effect is still debated but there is some evidence of NLRP3 inflammasome activation.

CPP AND PHOSPHATE TOXICITY

There is of course a lot of epidemiological evidence of an association between higher serum inorganic phosphate concentrations and mortality (particularly cardiovascular) in patients with renal disease and in the normal population [34]. This association, together with *in vitro* evidence obtained by adding phosphate to cells in culture, causing cellular dysfunction, suggested that extracellular phosphate may be a direct cellular toxin. Thus, for years we have concentrated on controlling serum phosphate concentrations in dialysis patients with increasing times on dialysis, dietary restriction of phosphate and prescription of phosphate binders (PBs) [35]. Such strategies are predicated on the hypothesis that high extracellular phosphate is toxic, and thus it is logical that reducing serum phosphate concentrations will by itself improve outcomes. However, there remain unexplained observations that make a phospho-centric paradigm difficult to fully accept. For example why is it that many mammalian species have serum phosphate levels that are far higher than those seen in humans, and if phosphate is so toxic, why does this not cause a problem. In fact, normal rat phosphate levels are some 0.5–1.0 mmol/L higher than in humans [36] despite being relatively calcification resistant, and the phosphate levels in some pigs are routinely >2–3 mmol/L [37]. Human neonates have phosphate levels between 1.0 and 2.8 mmol/L for the first few days of life. Additionally, in this paradigm it is hard to explain why 'phosphate toxicity' is abrogated almost completely by pyrophosphate, a compound that inhibits hydroxyapatite crystal formation and nanocrystalisation [38]. Phosphonoformic acid (also known as Foscarnet, an antiviral drug, and a pyrophosphate analog inhibiting viral DNA replication) also inhibits crystal formation at low concentrations [39] and abrogates calcification in a number of systems. This property has often been interpreted as being due to Pit1 inhibition (a sodium-phosphate cotransporter) and used as evidence to support a phosphate toxicity hypothesis but occurs at lower concentrations than those that affect this transporter [40]. Furthermore, if calcium is almost completely removed from the milieu [41] cellular dysfunction induced by phosphate is almost completely abrogated. In contrast, when almost serum-free media is used for cell culture, phosphate toxicity appears much worse, demonstrating a protective effect of serum components and suggesting that endogenous inhibitors of 'phosphate toxicity' are present naturally in the serum [38]. Sage *et al.* [38] showed that the addition of Fet-A can prevent such phosphate toxicity, with cellular dysfunction only occurring once nanocrystals are detected within the media. This suggests that calcium and phosphate ions may not in fact be the direct mediators of cellular toxicity by themselves at all, but more likely it is their product (calcium phosphate nanocrystals) that is the culprit. Thus, endogenous circulating inhibitors, which protect against this process (by forming CPP) may be far more influential on outcomes

than the concentrations of these inorganic minerals alone. Thus the more damaging CPP-II, which accumulate in disease, caused by enhanced formation or reduced removal, may be central to organisms attempts to prevent more widespread calcium phosphate deposition. This might explain some of the paradoxes and interpretation of much of the current data and show why relatively crude attempts to bind phosphate in renal disease have not elucidated a mechanism or been overwhelmingly successful in preventing death in controlled studies.

Winkelmayer *et al.* [42] studied mortality in 3603 incident patients starting dialysis. They were either prescribed calcium-based phosphate binders (CBPBs) (77.5%) or no binder [42]. Either prescribed CBPBS and the rest not taking binders. While the phosphate binder group had a lower overall mortality, when propensity score matched, no differences in mortality were evident and no differences in phosphate levels were observed between groups. In a prospective study of PBs in incident haemodialysis (HD) patients, Isakova *et al.* [43] examined a large (>10 000) cohort of incident HD patients treated or not treated with PBs within the first 90 days of starting dialysis. After adjusting for many known co-variates, mortality differences favoured those on PB therapy. However, these results were independent of baseline and follow-up serum phosphate concentrations. Cannata-Andía *et al.* [44] published similar results suggesting that phosphate binder use was associated with better survival on HD, but those patients on PB actually had higher serum phosphate levels [44] in the unadjusted data. When subgroup analysis was performed, the risk for subgroups appeared to be lower in all groups taking PB regardless of plasma phosphate level, even when plasma phosphate was low (<0.97 mmol/L). Interpretation of data like these does not make a compelling argument for targeting ever lower plasma phosphate levels, but perhaps suggests that if PBs do indeed reduce mortality, the mechanism of action may not be by phosphate lowering alone. Furthermore, this might well explain why calcium-based binders may be associated with higher risks than non-phosphate binders [45].

Thus, as we understand more about the drivers of soft-tissue mineralization, it is clear that crude attempts to reduce extracellular phosphate may actually play only a very small part in this process. Targeting CPP disruption, enhancing endogenous pyrophosphate formation or upregulating regulatory proteins to interfere with this process may be better targets for treatment. Calcium apatite nanocrystals within CPP2 may be far more important, and targeting their disruption, enhancing endogenous pyrophosphate formation or upregulating regulatory proteins may be better targets for therapeutic intervention.

TARGETING CPP

If we accept this model of free Fet-A playing a central role in health to protect against mineral nucleation and nanocrystallisation in the soluble phases, we can start to look at novel interventions using these pathways. At times when normal bone metabolism is perturbed, 'mineral stress' may occur due to an imbalance between production and removal of CPP locally. Such an imbalance might allow us to start to detect such particles in the vascular compartment. Fet-A also accumulates in membrane vesicles shed from vascular smooth muscle cells, which may be important in preventing further mineral nucleation. In patients with end-stage renal disease, the reduction of PTH by parathyroidectomy or calcimimetic also results in a reduction in serum CPP [28]. Free serum Fet-A levels are very low in calcific uraemic arteriopathy (CUA) with very high levels of secondary CPP [46], but with improvement in symptoms associated with removal of the circulating particles by plasma exchange [47], suggesting perhaps a role for CPP as biomarkers and potential mediators of CUA.

CONCLUSION

Fet-A is a key regulator of mineral trafficking, preventing nanocrystalline calcium apatite formation *in vivo* via the formation of CPP. Measurement of Fet-A protein and its interaction with mineral may provide some better biomarkers and targets for future therapies aimed at reducing soft tissue mineralization.

ACKNOWLEDGEMENTS

Thanks to A/Prof Eric Hanssen (Head, Advanced Microscopy Unit, Bio21, University of Melbourne) for technical assistance with the EM analyses presented in Figure 1. Funding has been obtained from the National Health and Medical Research Council (NHMRC) Australia, Jacquot Foundation and Royal Melbourne Hospital. The authors have also between them received research grants, honoraria or lecture fees from Amgen, Baxter, Gilead, Sanofi and Vifor Pharma. E.R.S. owns stock in Calciscon.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest with data presented within this review article.

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Received for publication: 1.2.2016; Accepted in revised form: 18.2.2016