Citation: BoneKEy Reports **4**, Article number: 672 (2015) | doi:10.1038/bonekey.2015.39

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REVIEW

The role of fetuin-A in mineral trafficking and deposition

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Calcium and phosphate are the principle ions involved in the deposition of mineral in the human body. Inhibitors of mineralisation are essential for the prevention of ectopic mineral precipitation and deposition. In the past decade, through *in vitro*, *in vivo* and clinical observation studies, we have come to appreciate the importance of fetuin-A (Fet-A), a circulating glycoprotein, in preventing ectopic calcium phosphate mineralisation. Moreover, the detection of Fet-A-containing mineral complex, termed calciprotein particles (CPPs), has provided new ways to assess an individual's calcific risk. The pathophysiological significance of CPPs in disease states is yet to be defined, but it provides an exciting avenue to further our understanding of the development of ectopic mineralisation.

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Introduction

The ability of the body to form calcified tissues is crucial to the maintenance of our structural integrity, but with mineralisation (or calcification) restricted to tissues such as bone, cartilage, dentin, cementum, enamel and otoconia. In bone and teeth, collagen I, the predominant protein in the extracellular matrix, serves as a scaffold onto which calcium (Ca), phosphate (Pi) and other ions crystalise as mineral apatite. This mineral–protein polymer gives rise to a unique material with excellent tensile and compressive strength.[1]

The concentrations of Ca and Pi in serum are supersaturated with respect to apatite. Therefore, one might expect that mineral nucleation and growth would occur in all extracellular fluid compartments, and thus the question with respect to biomineralisation is how the body regulates the process of crystal growth in bone and prevents crystal formation in extraosseous tissues. Failure to prevent ectopic mineralisation is common in conditions such as chronic kidney disease (CKD),[2,3] chronic inflammatory disease (CID)[4] and diabetes,[5] and it is also seen as part of the ageing process.[6]

The body utilises a variety of mechanisms to control mineralisation. Inorganic molecules such as magnesium and pyrophosphate (PPi), proteins such as albumin, matrix GLA protein (MGP), osteopontin and fetuin-A (Fet-A), and pH modulate Ca Pi precipitation both at a tissue level and systemically.[7–16] This review focuses on the liver-derived glycoprotein Fet-A (in humans known as α_2-Heremans Schmid glycoprotein) and its role as an important systemic inhibitor of extraosseous mineralisation.[17]

Mineralisation from a Physicochemical Perspective

It has been a long-held view that the crystal precipitation from a solution containing Ca and Pi follows that of the classical nucleation theory (CNT). This theory proposes that in a supersaturated solution random fluctuations in particle density result in the formation of tiny crystal nucleus of the same molecular structure as the final macroscopic crystal.[18] Crystal growth from the nucleus occurs via the addition of individual ions.

Recent studies using advanced imaging techniques such high-resolution cryo-transmission electron microscopy and atomic force microscopy have provided visual evidence of an alternative pathway to crystal nucleation, termed non-CNT (NCNT).[19–22] In contrast to CNT, NCNT suggests that stable pre-nucleation complexes (PNC) exist in supersaturated solutions. In the case of Ca and Pi crystallisation, the PNC is Ca triphosphate $(Ca(HPO_4)_3)^{4-}$ (**Figure 1**), and its concentration in solution is in an equilibrium with the concentration of Ca and Pi ions. PNC tends to aggregate to form larger aggregates, and, over time, these aggregates coalesce into amorphous Ca Pi (ACP), an unstable phase without crystalline structure.[22] The transformation (or ripening) from ACP to apatite (with a Ca:Pi ratio of 10:6) occurs with the further uptake of Ca ions and release of hydrogen ions. The final product, apatite $(Ca_{10}(PO_4)_6\bullet(OH)_2)$, is a thermodynamically stable crystal structure (**Figure 1**).

The speed in which crystals form in a supersaturated fluid such as the extracellular fluid depends on many factors. The degree of supersaturation has an obvious role, but inhibitors of

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Received 2 December 2014; accepted 13 February 2015; published online 6 May 2015

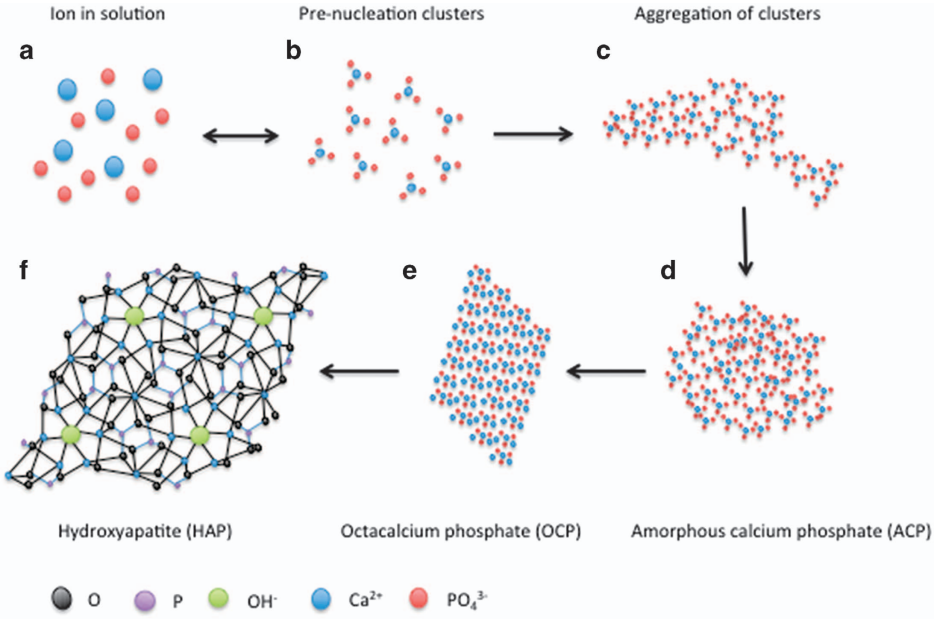

Figure 1 The pathway of hydroxyapatite formation. Adapted from Habraken *et al.*[22] with permission. A model of hydroxyapatite formation from PNC in solution. Free ions in solution (**a**) are in equilibrium with Ca triphosphate PNC (**b**). PNC aggregates (**c**), which then coalesce to become an ACP nucleus (**d**). With the addition of Ca ions and the loss of hydrogen ions, ACP transforms into OCP (**e**) and subsequently HAP (**f**), the most thermodynamically favourable state.

the crystal ripening process are equally important to control physiological mineralisation and to prevent ectopic mineralisation.

Fet-A Inhibits Ca Pi Crystal Growth

Proteins modulate crystal formation in at least two ways. Albumin for example, reduces the supersaturation of serum by binding free Ca ions to its acidic amino acid residues and EF-hand-like motifs. However, this process is relatively inefficient to prevent crystal precipitation, as evidenced by the fact that despite its abundance, albumin accounts for only ~50% of the mineralisation inhibitory activity of serum.[23] Rather than binding to individual ions, Fet-A inhibits Ca Pi crystal growth by binding to clusters of Ca Pi ions, acting as a barrier to further cluster aggregation.[24] The extensive computational modelling and mutant analysis work by the Jahnen–Dechent group has demonstrated that the β-sheet on the exposed surface of Fet-A is crucial to its ability to bind mineral.[24] This β-sheet contains regularly spaced negatively charged acidic amino acids in a lattice-like conformation.[24] Each acidic residue on the protein interacts with a Ca ion on the surface of a Ca Pi cluster, which reduces the available surface area for further cluster aggregation. In contrast to albumin, Fet-A circulates at around one-hundredth the concentration of albumin in adult human serum,[25] but it is at least 10-fold more efficient in its capacity to inhibit mineral precipitation in a supersaturated solution[24] (**Figure 2**).

Formation of Colloidal Calciprotein Particles

Fet-A inhibits Ca Pi precipitation in two steps. First, Fet-A binds to subnanometer-sized complexes of Ca and Pi, forming an entity termed calciprotein monomers (CPM).[26,27] If the solution remains supersaturated despite CPM formation, the thermodynamic drive for crystal nucleation remains and the ACP (see earlier) mineral phase emerges. At this point, it has been suggested that multiple Fet-A molecules, presumably through their binding with Ca ions, 'coat' the exterior surfaces of ACP to form primary calciprotein particles (CPP1).[26] This Fet-A 'shield' appears to stabilise ACP and retards its progression to more crystalline mineral phases. Over time, however, the particles aggregate and develop crystalline structures to form secondary CPPs (CPP2),[28] which have the mineral signatures of octacalcium phosphate or apatite[27,29] (**Figure 3**).

The ability of proteins to inhibit mineral growth by binding to Ca Pi clusters is not unique to Fet-A. Phosphorylated proteins such as osteopontin[30] and dentin matrix protein-1[31] are two examples of other proteins with apatite growth inhibitory activities *in vitro*, possibly via similar Ca Pi cluster binding mechanisms. Fet-A differs from these proteins in that protein phosphorylation is not required for its mineralisation inhibitory activity.[15,24,25] Furthermore, Fet-A appears to be the only such protein that accumulates in calcified tissue but which is not synthesised locally.[32,33]

Modulation of Skeletal Mineralisation by Fet-A

Given that at a molecular level Fet-A inhibits apatite formation and growth, it is somewhat counterintuitive that Fet-A has also been shown to *promote* collagen I matrix mineralisation in cell-free conditions. A series of experiments by Price *et al.*[34–36] have shown that serum alone can mineralise collagen I matrix, and such ability to induce matrix mineralisation is dependent on the presence of Fet-A in serum.[37,38] It is thought that Fet-A limits mineral formation outside collagen I matrix, constraining mineralisation to the small intrafibrillar space (0.3–0.6 nm in width) where only small molecules such as Ca and Pi can readily

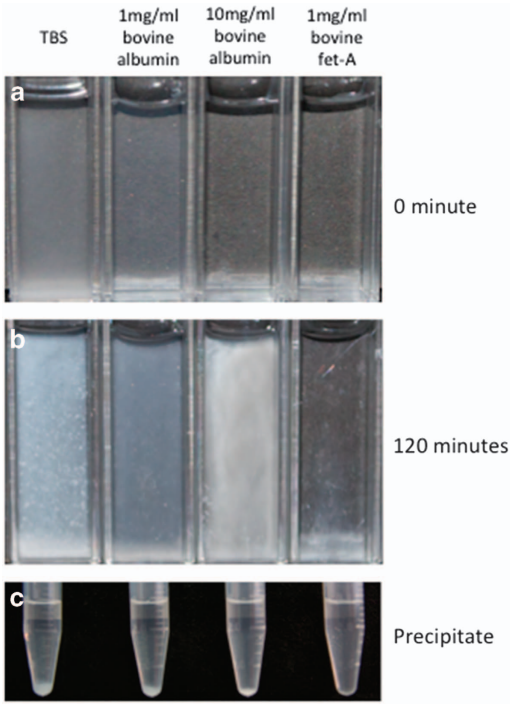

Figure 2 Fet-A is a potent inhibitor of Ca Pi precipitation in solution. Fet-A, fetuin-A. In a precipitation experiment, precipitation was instantly visible in Tris-buffered saline containing 10 mM $CaCl_2$ and 6 mM NaH_2PO_4 (**a**). After 120 minutes, the same solution spiked with 1 mgml^{-1} bovine fet-A remains transparent where as precipitates are visible in albumin solutions at 1mgml^{-1} and 10 mgml^{-1} (**b**). Aliquots of the solutions are then centrifuged at 1500g for 5 minutes at 4°C, pellets are visible in all solutions except for the fet-A containing solution (**c**).

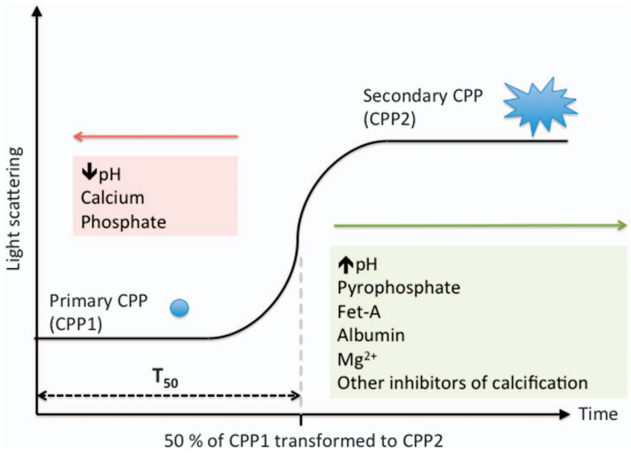

Figure 3 Formation and transformation of CPPs in a supersaturated solution. CPP, calciprotein particle; Fet-A, fetuin-A. Adapted from Pasch *et al.*[101] with permission. In the T_{50} test, a fixed amount of Ca and Pi stock solution is added to serum to induce supersaturation (10 mM Ca^{2+} and 6 mM PO_4^{3-}). Small (~60 nm), amorphous and spherical particles (CPP1) appear immediately but are only transiently stable. After a lag period, CPP1 transforms to larger and more crystalline particles (CPP2). T_{50} measures the time taken for light scattering intensity to reach its half-maximal read out. Calcification inhibitors delay the transformation from CPP1 to CPP2 (green box), substrates of apatite formation and acidosis accelerates this process (red box).

access. The fluid within the intrafibrillar space remains supersaturated, but in the absence of a potent inhibitor mineral precipitation is strongly favoured.[34]

In cell culture, Fet-A has been shown to inhibit osteoblast apoptosis by preventing apatite formation. These experiments used a serum-free 'osteogenic' medium containing supra-physiological Pi concentration.[15] The high Pi concentration in culture media without the presence of inhibitors of mineralisation results in the formation of apatite crystals that are cytotoxic,[29,39,40] possibly by activation of inflammasome pathways.[41] Therefore, in an *in vitro* 'osteogenic environment', Fet-A appears to promote cell survival by inhibiting apatite formation in the extracellular fluid. Although Fet-A may prolong survival of osteoblasts, it may also reduce osteoblast recruitment by inhibiting key signalling pathways of osteoblast differentiation from mesenchymal stem cells. Binkert *et al.* have shown that an Fet-A can bind to transforming growth factor-β and bone morphogenetic proteins (BMPs), cytokines crucial for osteoblastic differentiation.[42] At supraphysiological doses (30 μM, ~1.5 g l^{-1}), Fet-A completely inhibited osteoblast maturation.[42] In summary, these studies suggest that Fet-A may have different effects on *in vitro* mineralisation depending on the experimental conditions.

It would be hoped that an animal knockout model would help clarify some of the discrepancies from *in vitro* studies. However, Fet-A knockout models add further confusion to the interpretation of the cell-free and *in vitro* work. Fet-A knockout mice (*Ahsg*$^{-/-}$) have a normal skeletal phenotype at birth. *Ahsg*$^{-/-}$ adult mice, however, have shortened long bones with increased mineralisation of growth plates and cortical thickness.[43,44] Bone strength and mineral content are increased in *Ahsg*$^{-/-}$ in one study, but not in a subsequent experiment using animals from a different genetic background.[43,44] To further complicate the picture, Fet-A heterozygotes (*Ahsg*$^{+/-}$) did not exhibit an intermediate phenotype. In fact, reduction in serum Fet-A by two- to threefold reduced the mineralisation surface, the mineral formation rate and increased mineralisation lag time compared with wild-type or *Ahsg*$^{-/-}$ mice.[43] A possible explanation for the discrepancy between the cell-free experiments and *in vivo* findings is that the production of local mineralisation inhibitors such as osteopontin (OPN) is upregulated in the absence of Fet-A, which can substitute for Fet-A as a modulator of local mineralisation, allowing preferential crystallisation within collagen I matrix.[45] Taken together, the *in vivo* data suggest that bone mineralisation can occur in the absence of Fet-A with no significant compromise to its mechanical properties.

In clinical studies, the association of serum Fet-A concentration and bone mineral density (BMD) is also conflicting. As summarised in **Table 1**, higher serum Fet-A has been found to have either positive or no association with BMD, but no inverse associations have been reported thus far. The positive association between Fet-A and BMD is more convincingly demonstrated in women, and recent evidence suggests that oestrogen supplementation is associated with higher serum Fet-A levels,[46] but the direction of causality, if any, of this relationship has yet to be demonstrated. Furthermore, in a large prospective observation cohort of subjects >65 years, baseline plasma Fet-A was not predictive of fracture risk.[47]

Modulation of Ectopic Mineralisation by Fet-A

The spatial restriction of mineralisation to bone and teeth is sometimes lost in disease processes. Ca Pi deposits are commonly found within atherosclerotic plaques. In the elderly

Table 1 Studies with reported associations between serum Fet-A and BMD

Source	*Cohort*	N	*Association between BMD and serum fet-A*
Fink *et al.*[47]	Individuals aged >65 years	4714	No association with BMD after full multivariate adjustment.
Chailurkit *et al.*[103]	Healthy elderly women	82	High fet-A was associated with increased L2–4 BMD, but not associated with femoral neck BMD
Ix *et al.*[104]	Healthy individuals aged 70–79 years	508	High fet-A was associated with increased BMD across different sites in women. No significant association was detected in men.
Kirkpantur *et al.*[105]	Haemodialysis patients	72	High fet-A was associated with increased BMD across different sites except for lumbar vertebrae.
Fiore *et al.*[106]	Patients with established atherosclerosis	90	No association with BMD
Wilund *et al.*[107]	Sedentary older adults	12	No association with BMD
Avila *et al.*[108]	Prevalent female dialysis patients	197	Patients with a Tscore > -1.0 had higher fet-A compared with those with a Tscore $\leqslant -1.0$.
Sari and Uslu[109]	Postmenopausal women	90	Positive association with lumbar and femoral BMD
Sritara *et al.*[110]	Healthy volunteers	1741	No association with BMD

Abbreviations: BMD, bone mineral density; fet-A, fetuin-A.

and in patients with CKD, diabetes and CID, ectopic mineralisation is often present in the tunica media of arteries. In patients with calciphylaxis, mineral deposits can be found also in the skin, fat and other soft tissues. Histological examinations have shown that Fet-A is intimately associated ectopic Ca Pi deposits. This is seen in calcified arterial wall,[48-52] in ectopic joint mineralisation[53] and in calcified breast implant capsules.[54] Fet-A is notably absent however, in uncalcified areas, even in patients with end-stage kidney disease.[48,50]

Similar to osteoblast cell culture, vascular smooth muscle cell (VSMC) culture matrix mineralisation can be induced by supraphysiological concentrations of Ca and Pi ions.[48] Under these conditions, Fet-A has been shown to inhibit such mineralisation in a dose-dependent manner.[48,50] A reduction in the number of calcific nidi from apoptotic bodies and inhibition of mineral growth of matrix vesicles released from VSMC are again thought to account for the reduction in matrix mineralisation under these conditions.[48]

The ability of Fet-A to inhibit ectopic mineralisation *in vivo* was demonstrated in mouse knockout models. *Ahsg*$^{-/-}$ mice on the calcification-prone DBA-2 genetic background resulted in extensive ectopic mineralisation affecting virtually all organs.[17,55] Similar extensive ectopic mineralisation involving the kidney, heart and lungs were seen in *Ahsg*$^{-/-}$ on the calcification-resistant C57BL/6 genetic background, when these animals were challenged with a high-Pi diet and either vitamin D supplementation[17] or with the induction of CKD through renal ablation.[56] With respect to vascular calcification, *Ahsg*$^{-/-}$ mice crossed with atherosclerotic-prone *ApoE*$^{-/-}$ mice developed increased aortic intimal mineralisation, but this was only apparent in nephrectomised mice fed a high-Pi diet.[45] Despite these impressive phenotypes, two features are worth further consideration. First, *Ahsg*$^{-/-}$ mice do not display an overt calcific phenotype unless combined or challenged with other pro-calcific factors. This is seen in *Ahsg*$^{-/-}$ mice with a calcification-resistant background in which no significant ectopic mineralisation was seen in the absence of a high-Pi diet. Although the DBA/2 *Ahsg*$^{-/-}$ mice exhibited spontaneous ectopic calcification without exogenous stimuli, the DBA/2 strain itself is deficient in PPi,[57,58] another potent inhibitor of mineralisation. This suggests that under physiological circumstances Fet-A is not absolutely required to inhibit extraosseous mineralisation, but its importance is evident when mineral metabolism is perturbed or when redundant mechanisms against ectopic mineralisation fail. The second important observation is that vascular medial mineralisation was not a prominent feature associated with *Ahsg*$^{-/-}$ mice, even in those with CKD fed a high-Pi diet.[17,45,56] The preference for intimal mineralisation in *Ahsg*$^{-/-}$ mice differs from the exclusive medial mineralisation seen in other calcification inhibitor knockout models such as the *Mgp*$^{-/-}$ mice,[59] and the PPi-deficient *ENPP*$^{-/-}$ mice.[60] The reason for these phenotypic differences is unclear at this stage, but it suggests that *Ahsg*$^{-/-}$ mice may be a more suitable model to study endothelial injury and arterial intimal mineralisation.

Association Between Fet-A and Adverse Outcomes

The use of serum Fet-A as a risk marker was first studied in a haemodialysis cohort[61] and subsequently replicated in patients on peritoneal dialysis and on patients with CKD, hypertension, diabetes and coronary heart disease (see **Table 2** for the respective studies and references). A reduction in serum Fet-A may reflect decreased synthesis and consequent reduced capacity to inhibit extraosseous mineralisation, but it may also reflect the degree of extraosseous mineralisation owing to a 'consumptive phenomenon' in which Fet-A is removed from the circulation and sequestered in areas of mineral deposition.[58,62] As summarised in **Table 2**, although the majority of studies suggest an inverse association between serum Fet-A and adverse outcomes, several studies find no relationship or even positive associations. The discrepancies may stem from the fact that serum Fet-A is raised in obesity and insulin resistance,[63] conditions that in themselves are associated with adverse outcomes.

Association Between CPPs and Adverse Outcomes

More recent data suggest that total immunoreactive serum Fet-A may not be the most clinically relevant measurement with respect to pathology.[64,65] Circulating Fet-A may exist as free protein measured in the circulation of normal adults with a concentration of $\sim 0.3\ mg\ l^{-1}$,[66] but in pathological conditions Fet-A is also bound to circulating CPPs,[25,64,66] which make up a variable percentage of the total circulating Fet-A pool.

Table 2 Clinical studies assessing the relationship between total serum Fet-A, vascular parameters and patient outcome (updated from Hamano *et al.*[64])

Source	*Cohort*	N	*Association between adverse outcomes and total serum fet-A*	
			Univariate association	*Significant after MVA?*
Kettler *et al.*[61]	HD patients	312	Low serum fet-A was associated with inflammation, cardiovascular and all-cause mortality	No
Mehrotra *et al.*[111]	Patients with type II diabetes	88	Low serum fet-A was associated with CACS in patients with diabetic nephropathy	Yes
Moe *et al.*[50]	Patients with ESKD	51	Low serum fet-A was associated with CACS, but not aortic calcification	NP
Stenvinkel *et al.*[112]	Incident dialysis patients	256	Low serum fet-A was associated with malnutrition, inflammation, atherosclerosis, cardiovascular and all-cause mortality	Yes
Wang *et al.*[113]	PD patients	238	Low serum fet-A was associated with valvular calcification	Yes
Honda *et al.*	Patients with ESKD	176	Low serum fet-A was associated with mortality	Yes
Hermans *et al.*[114]	Prevalent dialysis patients	131	Fet-A was inversely associated with increased APWV in univariate analysis	No
Jung *et al.*[115]	HD patients	40	Fet-A was not associated with CACS	No
Cozzolino *et al.*[116]	HD patients	115	Low fet-A was associated with increased CACS	Yes
Mori *et al.*[117]	Healthy subjects	141	Higher fet-A was associated with increased carotid artery stiffness	Yes
Russo *et al.*[118]	Pre-dialysis CKD patients	53	Low fet-A was associated with increased CACS	NP
Hermans *et al.*[119]	HD and PD patients	987	Low fet-A was a predictor of overall mortality	Yes
Ix *et al.*[120]	Patients with CAD	970	Low fet-A was associated with mitral annular calcification and with aortic stenosis in patients with diabetes	Yes
Ix *et al.*[121]	Stages 3–4 CKD patients	822	Fet-A was not associated with all-cause or cardiovascular mortality	No
Mikami *et al.*[122]	Patients with diabetic nephropathy	85	Fet-A was not associated with CACS	No
Shroff *et al.*[123]	Children on dialysis	61	Low serum fet-A was associated with higher APWV	Yes
Metry *et al.*[124]	HD patients	222	Low serum fet-A was associated with mortality	No
Zheng *et al.*[125]	African-American HD patients	17	Serum fet-A was inversely associated with CACS	NP
Hamano *et al.*[64]	Pre-dialysis CKD patients	73	Serum fet-A was not associated with CACS	NP
Lorant *et al.*[126]	Patients with type II diabetes	76	Lower serum fet-A was associated with increased prevalence of PAD in patients	Yes
Mori *et al.*[127]	Patients undergoing coronary angiography	92	Lower serum fet-A was associated with CAC	Yes
Lim *et al.*[128]	Patients post acute myocardial infarction	754	Lower serum fet-A was associated with poorer 1 year survival	Yes
Marechal *et al.*[129]	Renal transplant recipients	277	Lower serum fet-A was associated with aortic calcification and cardiovascular events	Yes
Pateinakis *et al.*[130]	Haemodialysis patients	81	Lower serum fet-A was independently associated with increased APWV but not cIMT	No
Guarneri *et al.*[131]	Patients with essential hypertension	105	Lower serum fet-A was independently associated with increased cIMT	Yes
Scialla *et al.*[90]	Incident dialysis patients	602	The lowest serum fet-A tertile was associated with a significant increase in cardiovascular mortality risk	No
Jung *et al.*[132]	PD patients	67	Lower serum fet-A was independently associated with increased APWV	Yes
Roos *et al.*[133]	Patient post acute coronary syndrome	1049	Serum fet-A was not predictive of subsequent cardiovascular events	No
Emoto *et al.*[134]	Type II diabetes without significant renal impairment	416	Patients with calcified carotid plaques had lower serum fet-A compared with those without calcified carotid plaques.	Yes
Rittig *et al.*[135]	Patients at an increased risk of diabetes	315	Plasma fet-A was positively associated with cIMT.	Yes
Jensen *et al.*[136]	Elderly (>65 years old) subjects	3810	Higher serum fet-A was associated with lower incident cardiovascular disease only in non-obese individuals.	Yes
Kaess *et al.*[137]	Community cohort without pre-existing CVD	1870	Fet-A was not associated with CACS	No
Ford *et al.*[138]	Non-diabetic stage 3 and stage 4 CKD patients	73	Lower plasma fet-A predicted increase in APWV after 1 year	Yes
Smith *et al.*[65]	Stage 3 and stage 4 CKD patients	184	Total serum fet-A was not predictive of all-cause mortality	No

Abbreviations: APWV, aortic pulse-wave velocity; CACS, coronary artery calcification score; CAD, coronary artery disease; cIMT, carotid intimal media thickness; CKD, chronic kidney disease; CVD, cardiovascular diseases; ESKD, end-stage kidney disease; fet-A, fetuin-A; HD, haemodialysis; MVA, multivariate analysis; NP, not performed; PAD, peripheral artery disease; PD, peritoneal dialysis.

Owing to the higher density and size of CPPs, Fet-A bound to CPPs is readily detectable by differential centrifugation, or by ultrafiltration of body fluids.[25,64] Circulating CPPs in serum were initially discovered in etidronate-treated rats with profound hypercalcaemia (4.3 mM) and hyperphosphataemia (5.1 mM).[67] A high-molecular-weight species in serum containing Fet-A, MGP, Ca and Pi was detected by the above-mentioned methods.[67] Subsequent studies have demonstrated that a similar high-molecular-weight species containing Fet-A (without MGP) was present in the serum of rats with adenine-induced renal failure[68] and in the sera of patients with CKD and CID,[25,66] but it is notably undetectable in the serum of normal adults.[25,64,66] Interestingly, especially high levels of CPPs were recorded in dialysis patients with calciphylaxis.[66,69] We characterised the morphology of these particles in serum of dialysis patients as CPP2.[29] Although it is conceivable that in the

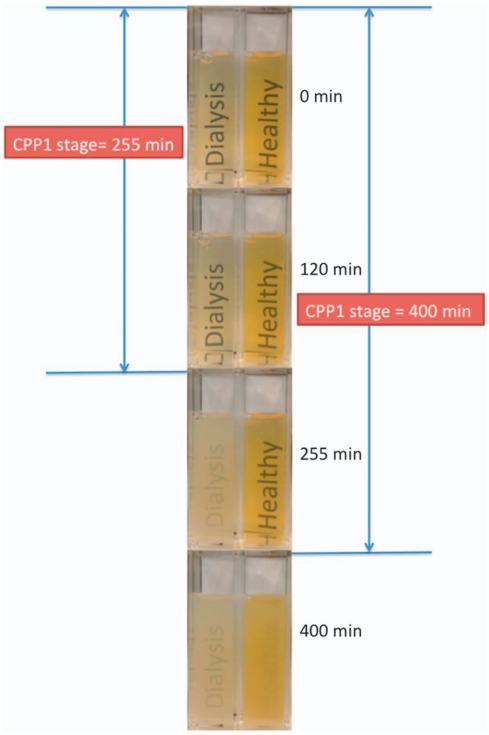

Figure 4 A conceptual representation of the T_{50} test. CPP, calciprotein particle; T_{50}, lag time. Protocol adapted from Pasch *et al.*[101]. To form a precipitation mix, Ca and Pi stock solution was added to serum of a healthy volunteer (right) and a dialysis patient (left) and incubated at 37°C. A standard flash enabled camera was used to capture the images obtained at the given time intervals. CPP1 stage appears translucent due to a relatively small degree of light scattering. After 255 minutes, the dialysis patient sample becomes opaque, indicating that CPP1 has transformed into CPP2, but the sample from the healthy volunteer remained translucent until 400 min, when it underwent a similar change in opacity.

aforementioned animal models CPPs may form in the intravascular space as a result of very high Ca and Pi concentrations, patients with moderate CKD and CID often have Ca and Pi concentrations within their respective reference intervals where spontaneous *in situ* formation in serum seems improbable. The origin of circulating CPPs remains unknown, but the fact that bisphosphonates, parathyroid hormone, vitamin D and osteoprotegerin are associated with serum CPPs[64,70] suggests that bone is the most likely candidate. CPP-like species have also been detected in the spent dialysate of some patients undergoing peritoneal dialysis.[28,71]

In CKD, serum CPPs are associated with coronary artery calcification,[64] increased aortic stiffness[25] and is predictive of all-cause mortality.[65] The well-known cardiovascular risk factor of inflammation becomes important here, as the independent predictive power of CPPs is attenuated, when high-sensitivity C-reactive protein is entered into the multivariate model.[65] This is interesting, as CPPs are also detected in patients with inflammation but normal renal function,[72] implying that inflammation *per se* may be important in the formation of CPPs.

Are CPPs Pathogenic?

Although the association between circulating CPPs and diseases is interesting, whether these particles are themselves pathogenic is unclear. Existing publications suggest that the core component of CPPs, Ca Pi nanocrystals, can be toxic when applied to cultured cell lines.[10,40,73–85] Furthermore, the nanocrystal shape and size modulated their cytotoxic effects.[40,76–78] It is important to point out that the particles used in these studies were synthesised in a protein-free environment, but the CPPs, as detected in serum, are synthesised in a protein-rich environment *in vivo*.[25,64] Theoretically, proteins can modulate the cytotoxicity of Ca Pi nanocrystals in at least two ways. Proteins such as Fet-A reduce aggregation and ripening of nanocrystals, thereby reducing their cytotoxicity. In addition to a given protein's physicochemical effects on crystal formation, the intracellular uptake of proteins, compared with unbound protein, is enhanced when proteins are bound to these nanocrystals.[86] The intracellular fate and effects of these proteins bound to CPPs is uncertain, but it is possible that they may exert additional effects on cells.

Murine studies have shown that cells of the reticuloendothelial system remove CPPs from the circulation partly via the cell surface class A scavenger receptor.[86] Macrophage uptake studies suggest that CPP-associated Fet-A is taken up much more readily than free Fet-A, suggesting that Fet-A is not the ligand responsible for macrophage endocytosis.[29,86] It is possible that other proteins associated with CPPs, such as OPN, may facilitate macrophage uptake of these particles.[87,88] Regardless of the exact mechanism of cell entry, CPP-induced proinflammatory cytokines release at high doses,[29,39] although the 'Fet-A shell' appears to dampen the inflammatory response to otherwise naked Ca Pi nanocrystals. Similar results have been replicated in VSMC culture.[10] It is therefore an oversimplication to categorise CPPs as 'good' or 'bad' particles. *In vitro* and clinical data suggest that the presence of CPPs (compared with its absence) is associated with cytotoxicity and adverse clinical outcomes, but this is likely an effect mediated by its mineral core. The protein components, on the other hand, actually protect cells from harmful effects of the mineral core.

Are CPPs the Mediators of *in vitro* 'Pi Toxicity'?

There is strong epidemiological evidence to suggest that high serum Pi is associated with adverse outcomes in patients with CKD.[89–91] The importance of Pi in mediating adverse outcomes is backed by observations that increased Pi intake induces vascular calcification in animal models.[17,56,92,93] *In vitro* experiments have also suggested that increased extracellular Pi may induce VSMC to express markers associated with osteoblastic/chrondrocytic-like cells, possibly via the Type III sodium-dependent Pi co-transporter (Pit-1).[94] It is worth considering that the effect ascribed to a high extracellular Pi in *in vitro* studies may be partly or entirely owing to the effect of CPPs that form in this environment, rather than free Pi ions *per se*. Increased Pi concentration in cell culture medium is often achieved by adding a small volume of a concentrated stock Pi solution. The high Pi concentration at the point of contact between stock Pi solution and medium may result in the nucleation of Ca Pi mineral phases and subsequent formation of CPPs. Indeed, CPPs can spontaneously form in cell culture medium after prolonged incubation,[95] and the addition of Pi (and Ca) would only serve to hasten the formation of CPPs in cell culture environments.[96] This phenomenon has been experimentally verified by Sage *et al.*, reporting that the addition of Pi resulted in nanocrystal formation with morphology similar to

CPP1. The cellular effects of 'high Pi' such as increased BMP-2 and OPN synthesis is primarily mediated by the effect of nanocrystals on cells.[97] Furthermore, experiments often use a Pit-1 antagonist, phosphonoformic acid (PFA), to demonstrate that reducing intracellular Pi uptake in response to high extracellular Pi can reverse the cellular effects of high extracellular Pi. However, PFA is also a potent inhibitor of Ca Pi crystal formation.[98] Therefore, the effect of PFA on cells could be also mediated via its inhibition of crystal formation, rather than via its blockade of Pit-1. In other *in vitro* work, increased Pi has been shown to induce VSMC autophagy[99] and endothelial apoptosis;[100] it is possible that these effects are also meditated via the formation of CPP *in vitro*. Future studies are needed to distinguish the cellular effect Pi ions from those of CPPs generated *in vitro*.

Serum Calcification Propensity

In parallel to the work on the biological effects of CPPs, understanding the mechanics of CPP formation has also paved the way to the development of a novel test, serum calcification propensity or the T_{50} test.[101] Biomarkers in routine clinical practice frequently rely on the presence and concentration of a particular substance. Functional assays, however, are more appropriate in some instances, and an example of this is the prothrombin time (PT). PT assesses the time taken for a plasma sample to clot after the addition of thromboplastin, an activator of the extrinsic coagulation cascade. PT is therefore a functional study assessing the cumulative effect of the various pro- and anti-coagulants in plasma. A similar concept, based on CPP biophysics, has been developed to determine the ability of the serum to resist crystal apatite formation. When large amounts of Ca and Pi are added to serum, CPP1 are formed. The lag time between the formation of CPP1 and its transformation to CPP2 is dependent on a number of parameters (**Figure 3**): pH, temperature and the concentration of Fet-A, Ca and Pi ions.[28,65,101] In a complex environment such as the extracellular compartment, the duration is also prolonged by the presence of other inhibitors of Ca Pi crystal growth such as PPi and magnesium ions.[65,101]

The lag time (T_{50}) is the time taken for 50% of CPP1 to transform into CPP2, as determined by their difference in size and ability to scatter light. Thus, T_{50} is analogous to PT in that it does not give information about any single factor, but rather reflects on the overall balance between pro- and anti-calcific factors in serum; that is, longer lag time suggests that the serum contains lower levels of pro-calcific factors or more anti-calcific factors.[65] The difference in light scattering from CPP1 to CPP2 can be detected via either three-dimensional dynamic light scattering or by nephelometry.[28,101] A photographic representation of the concept is shown in **Figure 4**.

The advantage of this assay is that it captures various known (and possibly unknown) factors that modulate mineralisation. We have shown that in a pre-dialysis CKD cohort T_{50} is inversely associated with known promoters of mineralisation such as ionised Ca and Pi, but it is positively associated with inhibitors of mineralisation such as PPi and serum Fet-A.[65] In the same cohort, a lower baseline T_{50} is associated with increased aortic pulse-wave velocity and increased inflammatory markers. Furthermore, a lower T_{50} was found to predict all-cause mortality, even after adjustment for routine clinical and biochemical parameters.[65] Similar results have recently been replicated in renal transplant recipients.[102] The T_{50} assay has the potential to revolutionise the assessment of 'calcific risk', but its availability is limited and not completely physiological, as it relies on adding large amounts Ca and Pi ions to reliably induce a highly supersaturated environment.[101] It is also important to note that unlike the serum CPPs detected in patients with CKD and CID, the CPPs generated in the T_{50} assay represent a 'test tube' phenomenon.

Summary and Conclusions

Through evolution, vertebrates have developed sophisticated mechanisms to restrict physiological mineralisation to bone and teeth. Fet-A is one of a number of overlapping factors that limit Ca Pi mineralisation in extraosseous tissues, while permitting physiological ossification. The study of Fet-A and its interaction with mineral has revealed a new paradigm to understand and assess the effect of disturbed Ca and Pi metabolism. Ectopic mineralisation is found in many disease states, but it is especially prevalent in those with CKD. Although the mainstay of therapy of ectopic mineralisation is currently focused on controlling serum Ca and Pi levels, finding ways to support inhibitors of mineralisation may be of equal importance in the treatment of unwanted mineralisation.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

MMXC is a recipient of a joint National Health and Medical Research Council (NHMRC) and Jacquot Foundation Postgraduate Research Scholarship. ERS has received research funding from Monash University, The Royal Melbourne Hospital Foundation, Amgen and Baxter, honoraria from Shire, and served as a consultant for Vifor Pharma. SGH has received research funding from the Jacquot Foundation, the Kinkaid-Smith fund and research funding or honoraria from Amgen, Baxter, Gilead, Novartis and Shire. The opinions expressed by this article are solely those of the individual authors and do not reflect the views of NHMRC or the Jacquot Foundation.

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