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REVIEW

The formation and function of calciprotein particles

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Abstract
Vertebrate extracellular fluids lie below the threshold for spontaneous calcium phosphate ($Ca–P_i$) precipitation; yet, they remain supersaturated enough to foster crystal growth if unchecked. Calciprotein particles (CPP) and their smaller precursor calciprotein monomers (CPM) have emerged as fast-acting "mineral buffers" that mitigate abrupt local oversaturation. Although these complexes typically contain only trace amounts of $Ca–P_i$ relative to total plasma levels, they exhibit remarkably high turnover kinetics, with clearance from the circulation within minutes, far outpacing hormonal loops that operate on timescales of hours to days. By forming ephemeral colloidal assemblies, CPM/CPP help maintain fluid-phase stability and avert uncontrolled crystallization "accidents" in microenvironments such as the intestine or bone-remodeling sites. However, under chronic mineral stress, such as in chronic kidney disease, multiple inhibitory factors (e.g., fetuin-A, pyrophosphate) can become deficient, enabling persistent generation of more advanced, crystalline CPP species. These "modified" CPP can adsorb additional ligands (e.g., apolipoproteins, microbial remnants, growth factors) and have been linked to inflammatory and pro-calcific changes in vascular and immune cells. Despite their minor quantitative contribution, these rapidly mobilized colloids may exert outsized influence on vascular and skeletal homeostasis, underscoring the need to clarify their origins, biological roles, and potential therapeutic targeting in disorders of mineral metabolism.

Keywords Calciprotein particles · Fetuin-A · Mineralization · Phosphate · Chronic kidney disease · Mineral stress

Overview—mineral buffering and mineral stress

Over the past decade, calciprotein particles (CPP) and their precursor calciprotein monomers (CPM) have increasingly been viewed as specialised "mineral buffers" in vertebrate plasma, mitigating abrupt surges in calcium and phosphate ($Ca–P_i$) [15, 50, 55, 95]. Conceptually, this function resembles how pH buffers stabilize hydrogen ion levels. Yet, this analogy is not exact, whereas classic buffers rely on reversible ionic binding to maintain equilibrium, CPP defends against the rapid nucleation of solid mineral phases through a colloidal, kinetic mechanism that is neither fully reversible nor strictly stoichiometric. Indeed, estimates of the CPM/CPP pool size challenge the idea that it functions as a bulk reservoir. While these complexes contain $Ca–P_i$ at low micromolar levels, overall plasma phosphate is present at millimolar concentrations.

Nevertheless, rodent studies where supraphysiological CPM/CPP are injected show that these colloids clear the bloodstream in minutes [49, 60, 63], compared with the ten-times-daily turnover of the exchangeable phosphate pool [66]. This disparity highlights that even though CPP make up only a negligible fraction of total mineral, they represent a high-activity, fast-turnover pool capable of addressing local oversaturation on a minute-by-minute timescale, a role hormonal loops cannot fulfil so quickly. They thus function not as "bulk carriers" of $Ca–P_i$ but as near-instant "first responders" forestalling crystallization "accidents" in transient or localized surges.

Such heightened $Ca–P_i$ activity can be termed *mineral stress* [121, 125], an overload of mineral ions that challenge the body's capacity to keep them in safe, soluble states. Under normal conditions, transient CPM/CPP can buffer these surges and clear quickly. However, in disease contexts such as chronic kidney disease (CKD), mineral stress may become persistent or repetitive. The repeated oversaturation, coupled

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with deficiencies in natural inhibitory factors (e.g., fetuin-A, pyrophosphate, magnesium), pushes the system to generate more advanced, crystalline CPP forms. Emerging data link these stable, "modified" CPP particles to vascular inflammation, endothelial injury, and ectopic calcification, underscoring the pathological potential of these rapidly mobilized, though quantitatively minor, colloids when mineral stress overwhelms normal clearance.

Calcium phosphate nucleation and crystallization

Calcium phosphates in mammals are most commonly associated with hard tissues (bones and teeth) and certain transient or extraosseous calcifications. Although the predominant phase in mammalian hard tissues is a non-stoichiometric substituted form of hydroxyapatite (HAP), several other calcium phosphate phases are also known to occur. For example, amorphous calcium phosphate (ACP), octacalcium phosphate (OCP), brushite (DCPD), and monetite (DCPA) can appear transiently during mineralization, in specific microenvironments, or under pathological conditions. Additional phases, such as tricalcium phosphates (α-TCP, β-TCP) and tetracalcium phosphate (TTCP), may also be present in pathological calcifications. Above pH 7.0, all these compounds, except for HAP, are unstable. In an aqueous environment, they will recrystallize or undergo solid-state conversion, ultimately forming HAP as the thermodynamic end product.

Under physiological conditions, the construction of an 18-ion HAP unit cell ($Ca_{10}[PO_4]_6[OH]_2$) from isolated ions is thermodynamically improbable. Instead, HAP formation is increasingly understood to proceed through a multi-stage, non-classical pathway in which stable or metastable ion complexes and amorphous phases precede the appearance of a well-defined crystalline lattice [36]. Initially, calcium and phosphate species associate into small pre-nucleation clusters (often referred to as PNCs or Posner-type clusters) whose exact composition, whether $Ca_9(PO_4)_6$ as initially proposed by Posner and Betts in the 1970s [7], $[Ca(HPO_4)_3]^{4-}$ [40], or other variants, remains an active topic of investigation. Neighboring clusters, bridged by ancillary phosphate molecules [69], can aggregate, condense/densify, or undergo liquid-like phase separation to form ACP particles [18, 24, 29, 34, 40, 68, 69, 145, 146]. Subsequently, through ongoing deprotonation, rearrangement, and uptake of additional ions, ACP undergoes structural reorganisation into more ordered phases, such as OCP, before eventually transitioning into crystalline HAP [18, 56, 67, 142].

Metastability of the aqueous calcium phosphate system

Evolutionary processes have ensured that vertebrate extracellular fluids maintain a delicately poised, near-threshold state, rather than reverting to static chemical equilibrium. These fluids remain subject to continuous ion fluxes, metabolic energy demands, and cellular regulatory actions, so they are prevented from stabilizing at the lowest energy configuration. Cellular and biochemical regulators thus retain significant influence over mineral deposition or dissolution. By operating under non-equilibrium boundary conditions, organisms can rapidly adapt to local or systemic requirements, instead of becoming chemically "locked." This arrangement grants tissues and cells the real-time flexibility to direct mineral handling, ensuring controlled mineralization, tissue repair, and protective buffering.

Vertebrate plasma is deemed *metastable* with respect to Ca and P_i, in that it lies below the threshold for spontaneous precipitation, yet remains sufficiently supersaturated to drive crystal growth [86]. Specifically, plasma is undersaturated with respect to the initial Ca–P_i phase (ACP), but supersaturated relative to HAP. Critically, HAP crystals autocatalyze further crystal formation at Ca–Pi levels beneath typical plasma values. Unrestrained, the ~ 2 kg of HAP in adult bone would perpetually sequester mineral ions, resulting in rapid depletion of the ECF of calcium and phosphate at rates outpacing any possible repletion mechanism by the gut or kidney. Consequently, it is essential to moderate the crystallization process so that it does not proceed too quickly or extend too far. If early-phase mineral formation were not slowed or halted, all mineral phases would readily mature into HAP, whose low solubility is incompatible with the homeostatic demands needed to preserve ECF composition. Indeed, the presence of HAP within the body can be viewed as a potential threat to the existence of the organism itself.

To avert uncontrolled crystallization, organisms insulate nascent HAP crystals from direct plasma exposure. Bone surfaces are encased by cell layers and matrix proteins, drastically limiting the fraction of "bare crystal" surfaces. Concurrently, ECF contains multiple inhibitory macromolecules that impede crystal nucleation and growth. Collectively, these measures allow for a tightly regulated bone mineralization process rather than an unregulated, spontaneous precipitation of minerals.

Mineral chaperoning—CPM and CPP formation

Biology harnesses the multi-step process of calcium phosphate crystallization by providing *chaperones* to bind nascent clusters or ACP droplets, stalling crystal growth at precisely timed intervals [54]. This ensures skeletal mineralisation occurs only within collagen fibrils and matrix vesicles, with minimal direct interaction between full-fledged HAP crystals and the ECF. Meanwhile, the bloodstream profits from the same principle, stabilizing incipient nuclei as fleeting colloids, Ca–P_i precipitation is forestalled, and hazardous crystal seeds are removed or broken down before

they threaten normal ionic homeostasis. The prototypical chaperone in mammalian ECF is the liver-derived phosphoglycoprotein, fetuin-A.

As depicted in Fig. 1, fetuin-A's key mineral-binding region lies in its N-terminal cystatin-like domain (CY1), which carries multiple acidic residues (Glu, Asp) and phosphorylated serine sites appropriately spaced (6–10 Å) to accommodate ~ 1-nm-sized CaP_i PNCs [45]. Although small-angle neutron-scattering studies support the binding of Posner-type clusters [47], the exact structure and composition of the relevant cargo remain uncertain. Current evidence indicates that, at near physiological pH, the predominant ion clusters may be relatively calcium-deficient and enriched in HPO_4^{2-} rather than the classic Posner-type arrangement [72]. Under supersaturated conditions, each fetuin-A molecule can bind in the order of 100 or more such clusters, creating CPM [47]. It has been postulated that the flexible, highly phosphorylated, intrinsically disordered carboxy-terminal region (CTR) might modulate binding by exposing or stabilising acidic residues in CY1 [53, 104].

Under conditions of sustained supersaturation, CPM can aggregate and recruit additional plasma proteins, generating larger colloids known as CPP [46]. As recently revealed by liquid-phase EM [107, 108], initially, these

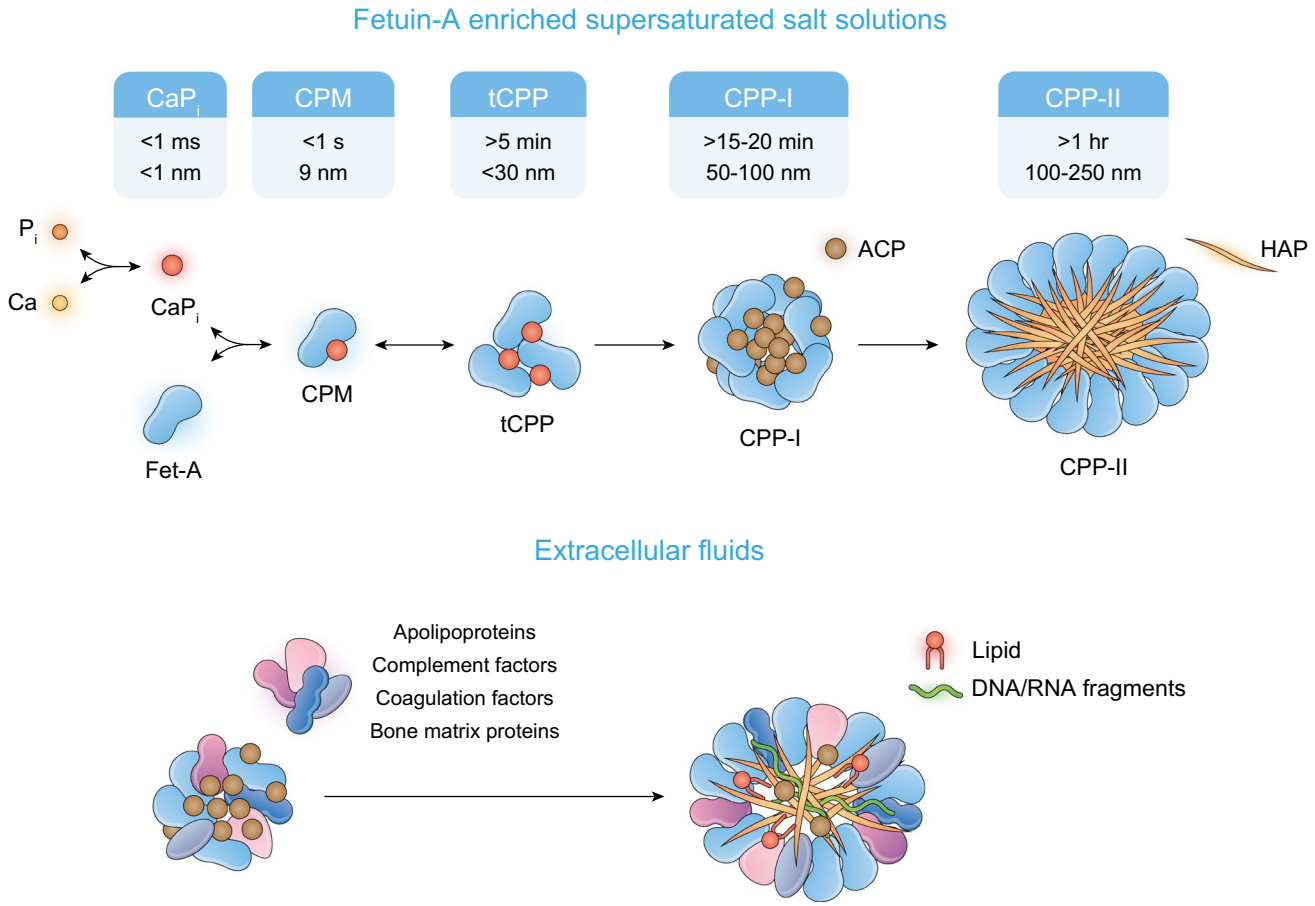

Fig. 1 Formation and maturation of calciprotein particles in fetuin-A-enriched supersaturated calcium phosphate solutions. Calcium (Ca) and phosphate (P_i) ions in near-physiological solutions can condense into sub-nanometer (~ 0.9 nm) ion clusters (CaP) that often display calcium-deficient stoichiometries. Bridging phosphate groups allows these clusters to aggregate. In biological fluids, mineral-binding proteins such as fetuin-A (Fet-A) can strongly interact with these pre-nucleation clusters, guiding them through nucleation, phase separation, and eventual crystal formation. The fundamental building block for calciprotein particles (CPP) is the calciprotein monomer (CPM), composed of CaP_i ion clusters bound and stabilised by monomeric Fet-A, primarily via acidic residues in its amino-terminal, cystatin-like domain 1 (CY1). Under sustained supersaturation, CPM molecules can consolidate into transient colloidal agglomerates (tCPP) before forming more stable, spherical (~ 30–100 nm) aggregates known as primary CPP (CPP-I), which carry amorphous calcium phosphate (ACP). In a final, irreversible step, the mineral phase reorganises into ordered, more thermodynamically stable crystalline phases, such as hydroxyapatite (HAP). Morphologically, these secondary CPP (CPP-II) are prolate ellipsoids measuring ~ 100–250 nm in length, containing densely packed needle-like lamellae of crystalline mineral. Approximate sizes and timescales are indicated. As shown in the bottom panel, the aggregation of CPM in extracellular fluids (e.g., serum) integrates additional proteins and molecular species, potentially conferring distinct biological properties. Compared to CPP formed in simple, Fet-A-enriched inorganic solutions, those generated in serum or other ECF, typically feature lower Fet-A and mineral content

assemblies may be transient and dynamic until equilibrium is reached, at which point larger spherical particles emerge, typically measuring ~ 50–100 nm in diameter. Each particle can comprise up to 100 CPM and contain diffuse ACP domains that occupy roughly 75% of its volume [45, 46]. These primary CPP (CPP-I) remain relatively stable for a few hours in vitro, provided that no additional destabilising factors are introduced. Over time, the amorphous core can reorganize into partially or fully crystalline mineral phases such as HAP. This transformation yields secondary CPP (CPP-II) [45, 46]. These typically measure 100–300 nm, with denser lamellar or needle-like crystalline regions resembling bone mineral platelets. Time-resolved TEM analyses indicate that the phase transformation follows a two-step process: first, the amorphous interior dissolves to form a hollow core; then, HAP crystallizes preferentially on the particle's surface (possibly involving changes in hydration), resulting in the more mature CPP-II morphology [45, 127]. The conversion reflects the thermodynamic drive for a stable crystal lattice and the kinetic conditions that allow ACP to persist long enough to rearrange. Once crystallized, such particles tend to clump further or sediment, partly due to the loss of zeta potential [127].

A host of physiochemical and biochemical factors modulate the transition from CPM to CPP-I and then to CPP-II. Lower pH, viscosity, ionic strength, higher temperature, and freeze–thaw cycles all favor particle aggregation and crystallization [78, 94]. Certain proteins like lysozyme also promote transformation. Conversely, other proteins, e.g., albumin; casein; phosvitin; and small molecules, magnesium, zinc, pyrophosphate, carbonate, and citrate, can inhibit crystal-phase transitions by binding exposed mineral surfaces [37, 94, 122, 132, 133], disrupting the ACP-to-HAP reorganization. Bisphosphonates, structurally mimicking pyrophosphate, reduce CPM aggregation and stall the amorphous-to-crystalline phase shift [3], suggesting both processes are tightly intertwined.

If generated in serum or other biological fluids, CPP acquire a "biomolecular corona" as they form and ripen, adsorbing not only additional fetuin-A or albumin but also complement factors, apolipoproteins, and a diverse array of distinct proteins, lipids (primarily cholesterol, phospholipids, and saturated fatty acids), and nucleic acids [127]. Although this might appear to be a nonspecific "serum-like" accumulation, in reality, the enrichment is selective: certain molecular classes (e.g., bone matrix proteins and omega-6 fatty acids) are conspicuously overrepresented, whereas others (like tetraspanins and plasma membrane markers) remain absent [127]. Notably, the ten most abundant proteins found in human CPP rank from first to 4330th in the serum proteome at large [127].

The conditional role of fetuin-A

Fetuin-A knockout mice show an elevated risk of ectopic calcification [109]; yet, this deficit does not uniformly or immediately trigger calcification events. Fetuin-A-depleted serum can still sustain the formation of CPP-like particles with added calcium and phosphate; although, the transition to crystalline forms proceeds considerably faster [94]. Unquestionably, fetuin-A critically influences the intermediate-term (minutes) stability of mineral colloids in extracellular fluid. However, for very short transit periods typical of CPM/CPP circulation, it may be dispensable if other stabilizers are present. Genetic backgrounds, defining the levels of companion inhibitors, e.g., magnesium and pyrophosphate, can mitigate or exacerbate the phenotype [6, 48]. Similar to a reported case of Caffey disease caused by a nonsense mutation in the *AHSG* gene leading to complete fetuin-A deficiency [76], fetuin-A null mice on a C57BL/6 background also exhibit skeletal abnormalities (e.g., epiphysiolysis associated with distal femur dysplasia and foreshortened hindlimbs) [11, 111]. By contrast, DBA/2 mice with low baseline magnesium and pyrophosphate experience aggressive intraluminal crystallization if fetuin-A is also removed [48]. These contrasting outcomes underscore that fetuin-A participates in an integrated inhibitor network, rather than acting as a single failsafe mechanism. CKD underscores this synergy and highlights the unfavorable result of bearing multiple hits to this system with falling fetuin-A and pyrophosphate levels, combined with rising phosphate levels, frequently tilt the system from safe ephemeral complexes towards more injurious forms of particles.

Kinetic constraints: in vitro vs. in vivo

Under laboratory conditions, extended incubation with saturating Ca–P_i will ensure that ACP eventually crystallizes, with substantial amounts of CPP-II forming (and even precipitating). Functional assays (like T50 measurements) highlight how quickly serum transitions to a crystal-laden state if forced, occurring in roughly 270 to 470 min, when 40% serum is supplemented to 10 mM Ca^{2+} and 6 mM phosphate at physiological pH and ionic strength [94]. Yet, actual bloodstream transit times rarely grant such extended ripening, since rapid clearance by kidneys or the liver (see below), combined with robust inhibition (fetuin-A, pyrophosphate, magnesium), generally keeps incipient amorphous complexes from crystallising extensively in vivo. Hence, many advanced forms captured in ex vivo assays could be artefacts of artificially maintained supersaturation or protracted handling [78]. High-grade pathologies, e.g., end-stage kidney disease (ESKD) may be needed before such advanced crystalline CPP consistently appear in circulation. In other words,

although thermodynamics favors stable crystalline phases, living systems impose stringent kinetic barriers that hamper or intercept that path.

The colloidal mineral phase in plasma

In purely inorganic solutions, spontaneous precipitation of solid Ca–P_i typically requires a $[Ca^{2+}] \times [P_i]$ product surpassing ~ 6 mM^2 (compared to the ~ 1.2–1.8 mM^2 of adult human plasma) [87]. However, boosting plasma Ca–P_i to that range rarely causes immediate precipitation, and values of ~ 16 mM^2 may be needed for an actual precipitate to form [90]. This gap is primarily explained by the formation of the colloidal mineral phase.

Eichholtz and Starling first inferred such a phase in 1925 when they observed that adding large amounts of neutral phosphate to defibrinated blood produced a non-diffusible colloidal complex of calcium phosphate that was impermeable to the glomerular membrane in their isolated kidney perfusion model [27]. Later, McLean and Hinrichs [73] confirmed that in dogs given phosphate intravenously or orally, a Ca–P_i colloid would rapidly form once the ion product exceeded ~ 3 mM^2. Injecting as much as 50 mmol of phosphate caused transient tetany and convulsions. In contrast, administering a preformed, mineral-loaded colloid—which had limited capacity to bind additional mineral ions—maintained stable ionised calcium levels, leaving the animals asymptomatic. Gersh independently replicated these findings in dogs and rats [38, 39], showing that macrophages in the liver and spleen were responsible for clearance. The formation of such colloidal calcium phosphate (initially presumed to be $CaHPO_4$) also explains the lowering of ionised calcium during intravenous phosphate infusions to treat hypercalcemia [44]. Radiolabeled strontium incorporated in these phosphate colloids likewise localizes to the liver for clearance [20, 28]. Some decades later, Price and colleagues "re-discovered" this colloid in adolescent, actively mineralizing, rats challenged with supra-therapeutic doses of etidronate [101]. As a result of etidronate overdosing (32 mg/100 g body weight), mineral ion flux originally destined for bone was diverted to the plasma, causing a large spike in serum calcium (> 9 mM) and phosphate (> 8 mM) within 6 h of injection. At this time point, approximately 80% of the plasma calcium and phosphate was recovered as a circulating, high-molecular-weight mineral-protein complex containing fetuin-A. Price termed these colloids "fetuin-A mineral complexes," but they are synonymous with the CPPs and other mineraloprotein particles variously described by different authors as bions, nanons, and calcifying nanoparticles (and even the now debunked notion of nanobacteria) [147].

Where and why CPM/CPP form: likely microenvironments beyond plasma

These observations demonstrate that plasma, which is abundant in mineralisation inhibitors, does not typically permit large-scale colloid generation under physiological conditions. Even in advanced CKD, where hyperphosphatemia is prevalent, the concentrations of Ca^{2+} and phosphate rarely, if ever, reach levels permitting spontaneous ACP nucleation. Instead, the key sites to consider are local microenvironments where transient ionic activity may briefly surpass these thermodynamic thresholds. Among these, the intestinal lumen and bone remodelling surfaces stand out as primary candidates. However, any interface involving substantial mineral ion flux across epithelia could be relevant. Indeed, various epithelial secretions like saliva, breast milk, alveolar surfactant, and seminal fluid are recognized to be rich in phosphate, conferring some inherent risk of incipient mineralization.

Intestine

Experimental data suggest that the intestine could be the major source of CPMs/CPPs. In particular, following a phosphate-rich meal, Ca–P_i levels in the small intestine lumen can transiently exceed solubility limits (phosphate > 30 mM in the distal ileum) [33, 51], promoting the nucleation of small mineral clusters. Notably, salivary (~ 4 mM) and biliary (~ 5 mM) secretions contain relatively high phosphate levels [4], implying that some degree of nucleation may occur even in the fasting state. Proteomic analyses indicate that fetuin-A is nearly undetectable in gastric and intestinal secretions [96, 102] (as expected, given the absence of local expression in the GI tract [8]). Nonetheless, CPP-I-like magnesium-substituted ACP nanoparticles appear abundant in the distal small intestine lumen [99], likely stabilized by alternative binding proteins and other environmental factors. These particles have been observed to reach Peyer's patches (immune cell–rich sites for particle scavenging) through epithelial M cells. Any particles that evade local clearance and enter the bloodstream through the portal or lymphatic pathways may subsequently bind to fetuin-A and other chaperones once they reach the systemic circulation.

Phosphate binder studies in dialysis patients illustrate how blocking or reducing intestinal phosphate uptake leads to marked decreases in endogenous CPP [84, 128, 134], suggesting that newly absorbed dietary phosphate can seed Ca–P_i colloids in the post-absorptive setting. In one randomized trial, high-dose sucroferric oxyhydroxide therapy significantly lowered CPM, CPP-I, and CPP-II levels, and the corresponding serum exhibited diminished pro-calcific and pro-inflammatory activity in vascular cell bioassays [134]. Recent feeding studies in healthy adults have demonstrated that a standardised

meal containing ~ 300 mg calcium and ~ 188 mg phosphate leads to marked postprandial increases in all circulating CPP subspecies long before any sustained change in total serum phosphate is detectable [136]. Moreover, in CKD patients with reduced kidney function, these responses are heightened, with fasting CPM levels already elevated. Studies in rodents fed high-phosphate diets also show increased levels of circulating CPPs [3, 42, 70, 126], whereas when dietary phosphate is restricted [42] or intestinal phosphate uptake is blocked [85], abundance in blood diminishes. These convergent data highlight that the intestine, under dietary phosphate loads, can briefly exceed local solubility thresholds, enabling Ca–P_i clustering that emerges systemically as CPM/CPP once it encounters fetuin-A and other stabilizers in plasma.

Bone

A growing body of investigations also directly connects changes in bone turnover to fluctuations in CPM/CPP levels, implying that active skeletal remodelling is another key source. Pharmacological interventions that modulate osteoclast or osteoblast function—such as administering bisphosphonates [100, 101], calcimimetics [135], or anti-inflammatory biologics [141], consistently show parallel changes in bone turnover markers and CPM/CPP in vivo. In a 28-day randomized trial of hemodialysis patients, raising dialysate magnesium from 1.0 to 2.0 mEq/L markedly lowered CPP-I and CPP-II and systemic inflammation while simultaneously decreasing tartrate-resistant acid phosphatase 5b (a marker of bone resorption) and increasing bone-specific alkaline phosphatase (a marker of bone formation) [9]. In another study of hemodialysis patients receiving the calcimimetic etelcalcetide, the decline in osteoclastic markers following PTH suppression was mirrored by robust reductions in CPM, CPP-I, and CPP-II, with this effect reversed upon withdrawal of therapy [135]. Similarly, in patients with chronic inflammatory disorders treated with infliximab, suppression of bone resorption (accompanied by improved bone formation) coincided with significant drops in CPM and CPP-I [141]. These data suggest that as osteoclasts release or mobilize Ca–P_i from bone, a portion may bind to chaperones (e.g., fetuin-A) and enter the circulation as CPM/CPP if it is not metabolized locally or re-incorporated into the matrix. Notably, although fetuin-A is not synthesized within bone, it is one of the most abundant non-collagenous proteins accumulated from plasma and incorporated into the matrix [25]. This local reservoir of fetuin-A may thus provide a proximal source for de novo colloid formation if it survives the degradative processes of bone resorption. Additional evidence from conditions like Fabry disease, where high CPP levels correlate inversely with hip bone mineral density [10], supports the premise that deficits in skeletal mineral retention may drive more Ca–P_i complexes into the blood. Careful preservation of in vivo structures using cryogenic-processing techniques has revealed abundant CPP-I-like particles in the extracellular space adjacent to the surfaces of growing long bones in chicken embryos [57]. Moreover, membrane-delimited vesicles containing these CPP-I-like particles have been observed in the lumen of blood vessels near developing bone [58]. While CPP and mineral-containing extracellular vesicles (e.g., matrix vesicles) are widely considered distinct entities, the removal or disruption of the vesicle membrane (i.e., "denuding" EVs) may provide a source of circulating CPP.

Distinguishing the relative contributions of bone and intestine to circulating CPP remains an area of active investigation. Both sources seem plausible, and their contribution may vary with physiological state. In adults, the intestine may play a more prominent role after a phosphate-rich meal since fasting levels of CPM and CPP are markedly lower than in the fed state [136]. Whether this is the case in ESRD patients or in instances of markedly aberrant states of bone turnover is unknown.

The key point is that these origins share a common theme: CPP formation likely occurs in specialized microenvironments characterized by higher ion activities, longer residence times, and perhaps fewer inhibitors than open circulation. In other words, CPPs do not simply "appear" in the blood; they leak, spill, or efflux into the bloodstream from compartments where mineral handling is more permissive. Thus, what we detect as "circulating" CPP might be a fraction of these extravascular creations that have found their way into the blood and have not yet been cleared locally or systemically.

Clearance pathways

Recent studies suggest that CPM and to a lesser extent, fetuin-A, are filtered at the glomerulus and subjected to handling by proximal tubule with reclamation via megalin-mediated endocytosis. Prior work has shown that megalin blockade (e.g., His-Rap–mediated inhibition or Clcn5 knockout) results in increased fetuin-A levels in the tubular lumen [71, 105], whereas inhibiting lysosomal proteolysis (e.g., with leupeptin) promotes fetuin-A accumulation within proximal tubule cells raising levels above the detection limit [71]. Interestingly, CPM are taken up more readily than monomeric fetuin-A [60], allowing easier detection in the absence of protease inhibition. Although most free fetuin-A is reclaimed via proximal tubule endocytosis, small quantities can appear in the final urine [137], likely reflecting both incomplete reclamation and/or active exosomal secretion by tubular epithelial cells [151]. This observation parallels similar low-level urinary losses of albumin and aligns with longstanding data suggesting minimal but physiologically relevant filtration of plasma proteins. The efficient filtration–reabsorption pathway for fetuin-A is further modulated by the neutral-to-slightly negative surface charge that mineral binding confers on CPM [60], reducing electrostatic

repulsion within the glomerular filtration barrier. Once in the primary urine, CPM may dissociate due to local changes in pH or ionic strength, followed by fetuin-A reabsorption and mineral excretion. Alternatively, intact CPM may be endocytosed in toto and subsequently dissociated in lysosomes.

Due to their size, CPP seldom, if ever, navigate through the glomerulus; instead, hepatic uptake, notably via liver sinusoidal endothelial cells (CPP-I) and Kupffer cells (CPP-II), dominates [63]. While the receptors for CPM and CPP-I have yet to be elucidated, in mice, CPP-II clearance appears to be partly dependent on the class A scavenger receptor [49], with apolipoprotein-A1 serving as the likely cognate ligand. Tracer studies in healthy rodents show blood clearance in minutes, illustrating a robust clearance capacity under normal conditions. Disposition and elimination of exogenous CPP-II also appear unaffected by CKD [149], suggesting that it is not a state of impaired clearance (at least acutely) as is often assumed. However, a caveat to these studies is using synthetic CPP as tracers since it is unknown whether native CPP, modified by the in vivo environment, may differ in their fate and clearance routes.

Phosphate sensing and endocrine regulation

Several lines of evidence indicate that CPMs, and to a lesser extent CPPs, may communicate rising dietary phosphate loads to bone and thus influence plasma phosphate control through established endocrine networks. In vitro data suggest that CPM can stimulate de novo expression and/or secretion of FGF23 in osteoblasts and osteocytes [3, 106], possibly through a pathway involving fibroblast growth factor receptors [3]. In rodents, a rise in circulating CPMs was observed to precede an increase in bone FGF23 mRNA and protein levels administered phosphate by oral gavage [3]. Indeed, in human feeding studies, CPM/CPP increase before an appreciable shift in plasma Pi and FGF23 levels [136]. Central to this proposition, it has been shown that CPM/CPP may extravasate from the vascular compartment into bone tissue [3], reinforcing the idea that they may act not merely as scavenged complexes but also as short-lived "messengers" carrying labile Ca–Pi to skeletal cells.

However, there is yet no evidence to show that exogenous CPM or CPP administration directly elicits FGF23 (or PTH) production in vivo. Data from fetuin-A knockout models, where CPM formation is essentially absent, also tempers enthusiasm since these mice have measurable FGF23 levels and retain the capacity for phosphate homeostasis, suggesting that CPM is not an indispensable secretagogue [6]. Collectively, these findings position CPM/CPP as a rapid-response component of phosphate handling: they emerge in response to transient surges, travel transiently in plasma, extravasate to bone, and potentially trigger FGF23, supplementing other putative P_i messengers.

A minor quantitative contribution, yet potentially outsized influence

Nano-flow cytometric quantitation of circulating (plasma/serum) CPP levels in various observational [10, 16, 32, 103, 122, 126, 136–138, 141] and interventional [9, 128, 134, 135, 139, 140] human studies—comprising healthy individuals, patients with CKD, and other chronic disorders—has revealed typical non-fasting (random) CPP-I and CPP-II levels of 10^5–10^7 and 10^3–10^5 per mL, respectively. Quantitatively, these levels are surpassed by several orders of magnitude by other circulating particulates, such as lipoprotein particles (10^{13}–10^{16} per mL) [19] and extracellular vesicles (~10^9–10^{12} per mL) [23]. A reasonable question to consider is how these particle numbers translate into the amount of mineral transported. Inductively coupled plasma–optical emission spectrometry (ICP-OES) analysis suggests that the CPP-containing fraction of plasma seldom exceeds 50 ng/mL (~1.2 µM) of calcium, accounting for < 0.05% of total plasma calcium (unpublished observations). Given the crude isolation method and potential contaminants [127], this value likely overestimates the true figure. However, consistent approximations emerge from assumptions about particle shape (spherical vs. prolate ellipsoid), mineral phase (stoichiometry and density), and volume/packing.[1] At more representative plasma concentrations, empirical calculations yield estimates for CPP-I (10^6/mL) of ~10 nM calcium and 6.8 nM phosphate and for CPP-II (10^5/mL), of ~6 nM calcium and 3.5 nM phosphate.

[1] Estimation of mineral content in CPP isolated from ESKD patients' plasma: plasma CPP-Ca = 50 ng/mL by ICP-OES; CPP-I 2.1×10^7/mL, CPP-II 2.7×10^6/mL by nano-flow cytometry.

CPP-I assumptions: shape/size: spherical 100 nm diameter; mineral phase and density: ACP ($Ca_3(PO_4)_2$), $\rho = 2.7$ g/cm^3, MW = 310.18 g/mol; volume occupancy: 75% (Heiss et al. 2003).

Volume of single CPP-I: $V_{\text{sphere}} = \frac{4}{3}\pi r^3 = \frac{4}{3}\pi \times 50^3 \approx 5.24 \times 10^5 \text{nm}^3$

Volume of ACP in single CPP-I:

$5.24 \times 10^5 \text{nm}^3 \times 0.75 = 3.93 \times 10^5 \text{nm}^3 (\times 10^{-16} \text{cm}^3)$

Mass of ACP per CPP-I: $3.93 \times 10^{-16} \text{cm}^3 \times 2.7 \frac{\text{g}}{\text{cm}^3} \approx 1.06 \times 10^{-15} \text{g}$

Mol of ACP per CPP-I: $\frac{1.06\times10^{-15}\text{g}}{310.18 \frac{\text{g}}{\text{mol}}} \approx 3.42 \times 10^{-18}$ mol ACP

Mol of Ca: $3.42 \times 10^{-18} \times 3 = 1.03 \times 10^{-17}$ mol Ca

Mol of P: $3.42 \times 10^{-18} \times 2 = 6.84 \times 10^{-18}$ mol P

Total mol of Ca in 2.1×10^7 CPP-I:

1.03×10^{-17} mol $\times 2.1 \times 10^7 = 2.16 \times 10^{-10}$ mol Ca (0.216 nmol).

Total moles of P in 2.1×10^7:

6.84×10^{-18} mol $\times 2.1 \times 10^7 = 1.44 \times 10^{-10}$ mol P (0.144 nmol).

CPP-II assumptions: prolate ellipsoid; long axis 300 nm × short axis 125 nm; HAP ($Ca_5(PO_4)_3OH$), $\rho = 3.16$ g/cm^3, MW = 502.3 g/mol; volume occupancy: 75% (Heiss et al. 2003).

Volume of single CPP-II:

$V_{\text{ellipsoid}} = \frac{4}{3}\pi ab^2 = \frac{4}{3}\pi \times 150 \times 62.5^2 \approx 2.45 \times 10^6 \text{nm}^3$

Volume of HAP in single CPP-II:

$2.45 \times 10^6 \text{nm}^3 \times 0.75 = 1.84 \times 10^6 \text{nm}^3 (\times 10^{-15} \text{cm}^3)$.

For CPM, the mineral load remains less certain. A theoretical maximal loading estimate can be derived on the basis of the concentration of fetuin-A in plasma (10 µM) and the capacity of each molecule to bind up to 100 Posner-type clusters $Ca_9(PO_4)_6$ [47]. This implies carriage of up to ~ 6 mM phosphate and ~ 9 mM calcium. Still, this evidently does not occur in vivo: total acid-soluble phosphate typically remains under 1.5 mmol/L, and > 90% of plasma phosphate is freely diffusible, with < 10% being protein-bound or complexed [91]. Logically, the mineral carried by monomeric fetuin-A must, therefore, be lower. In simple solutions (1.5 mM Ca, 0.8 mM P, and 10 µM fetuin-A), anion-exchange chromatography indicates that ~ 10–15% of fetuin-A is bound to mineral [60]. Nevertheless, in human plasma, less than 0.05% of fetuin-A appears in the CPM fraction (unpublished observations). Even if each of these fetuin-A molecules were loaded with 100 Posner clusters, this would only account for approximately 0.6 µM phosphate.

Based on these measurements and theoretical considerations, it seems reasonable to conclude that collectively, CPM and CPP constitute only a small portion of the total phosphate (and calcium) pool in plasma (Fig. 2). It is, therefore, unlikely that the circulating CPM/CPP pool serves as an indispensable mineral depot for mineralizing tissues. Indeed, fetuin-A knockout mice do not present an under-mineralized bone phenotype, but rather show accelerated/premature growth plate closure, leading to stunted forelimb growth [111]. Notably, the inherent instability of CPM and CPP-I means both readily decompose under standard calcium and phosphate assay conditions, meaning their ionic contribution is typically subsumed within conventional biochemical measurements. CPP-II is more resistant to chemical dissolution and only partially disaggregate to yield ionic components, but this underestimate is probably inconsequential.

Nonetheless, the earlier colloidal forms display rapid turnover and may spontaneously assemble and disband in local surges or spikes. As such, they may act primarily in states of transient supersaturation to swiftly sequester excessive minerals, stabilizing the fluid-phase composition. From an adaptive standpoint, if these colloids were abundant, it would suggest the system is struggling to maintain disequilibrium and their very scarcity in health implies that they accomplish their buffering tasks very efficiently and then are removed from circulation or disassembled. Consequently, they are better considered a "pressure relief value" rather than major conveyors of minerals, bridging acute local overshoot until comparatively slower endocrine responses restore homeostasis.

Neonatal cord blood exemplifies such adaptation; neonates require high phosphate (i.e., sustained positive balance) to drive skeletal growth; yet, they seldom display pathological mineralization. Here, CPM and CPP-I levels are much higher than in adults, with levels more akin to those observed in patients with ESKD [122]. Conversely, levels of crystalline CPP-II are very low, signifying enhanced activity stabilizing earlier colloidal forms and preventing transformation to the crystalline state. Intriguingly, the "usual suspects" (fetuin-A, magnesium, pyrophosphate) could not account for this enhanced resistance to crystallization pressure, so unknown other factors appear at work. Harnessing this knowledge could help inform strategies to bolster defences in more calcification-prone settings like CKD.

CPP-induced pathology and methodological considerations

The use of the T50 test in multiple observational cohorts over the past decade has consistently shown that accelerated conversion of CPP-I to CPP-II in serum correlates with an elevated risk of adverse cardiovascular and renal outcomes [12, 13, 22, 59, 93, 123, 148]. Although this phenomenon has been most studied in CKD populations, analogous findings in non-renal settings point to a broader applicability of these concepts [26, 75, 143]. This epidemiological backdrop has spurred considerable interest in whether CPPs might directly underlie some of the deleterious effects associated with dysregulated mineral metabolism or aptly described by Makoto Kuro-o, "the true culprit of phosphates woes" [65].

Seemingly, in vitro data now strongly support a pathogenic role for CPP, and especially CPP-II. These particles stimulate osteogenic transformation and mineralization in vascular smooth muscle cells (VSMCs) [1, 2, 17, 131, 150], inhibit osteoblast mineralisation [17], impair endothelial function by decreasing nitric oxide bioavailability [31, 114, 116–118, 129], and incite inflammatory cascades through TLR4 and IL-1β/IL-1α release, mediated by both NLRP3 inflammasome–dependent and –independent pathways in monocytes/macrophages [5, 52, 63, 82, 125]. Impaired myoblast differentiation [61] and tubular cell cytotoxicity [60, 64, 113] has also been observed.

Irrespective of cell type, the generic response to CPP internalization is typified by lysosomal disruption, oxidative stress, and pro-inflammatory signalling. Ex vivo studies

Footnote 1 (continued)

Mass of HAP per CPP-II: $1.84 \times 10^{-15}\text{cm}^3 \times 3.16\frac{\text{g}}{\text{cm}^3} \approx 5.82 \times 10^{-15}\text{g}$
Mol of HAP per CPP-II: $\frac{5.82\times10^{-15}\text{g}}{502.3\frac{\text{g}}{\text{mol}}} \approx 1.16 \times 10^{-17}$ mol HAP
Mol of Ca: $1.16 \times 10^{-17} \times 5 = 5.8\text{x}10^{-17}$mol Ca
Mol of P: $1.16 \times 10^{-17} \times 3 = 3.48 \times 10^{-17}$mol P
Total mol of Ca in 2.7×10^6 CPP-II:
$5.8 \times 10^{-17}\text{mol} \times 2.6 \times 10^6 = 1.51 \times 10^{-10}$ mol Ca (0.151 nmol).
Total moles of P in 2.7×10^6 CPP-II:
$3.48 \times 10^{-17}\text{mol} \times 2.6 \times 10^6 = 9.05 \times 10^{-11}$ mol P (0.091 nmol).
For total CPP-Ca (estimated): CPP-I (0.216 nmol) + CPP-II (0.151 nmol) = 0.367 nmol = 14.7 ng per mL.
Plasma CPP-$Ca_{ICP\text{-}OES}$: 50 ng/mL, plasma CPP-$Ca_{estimated}$: 15 ng/mL.

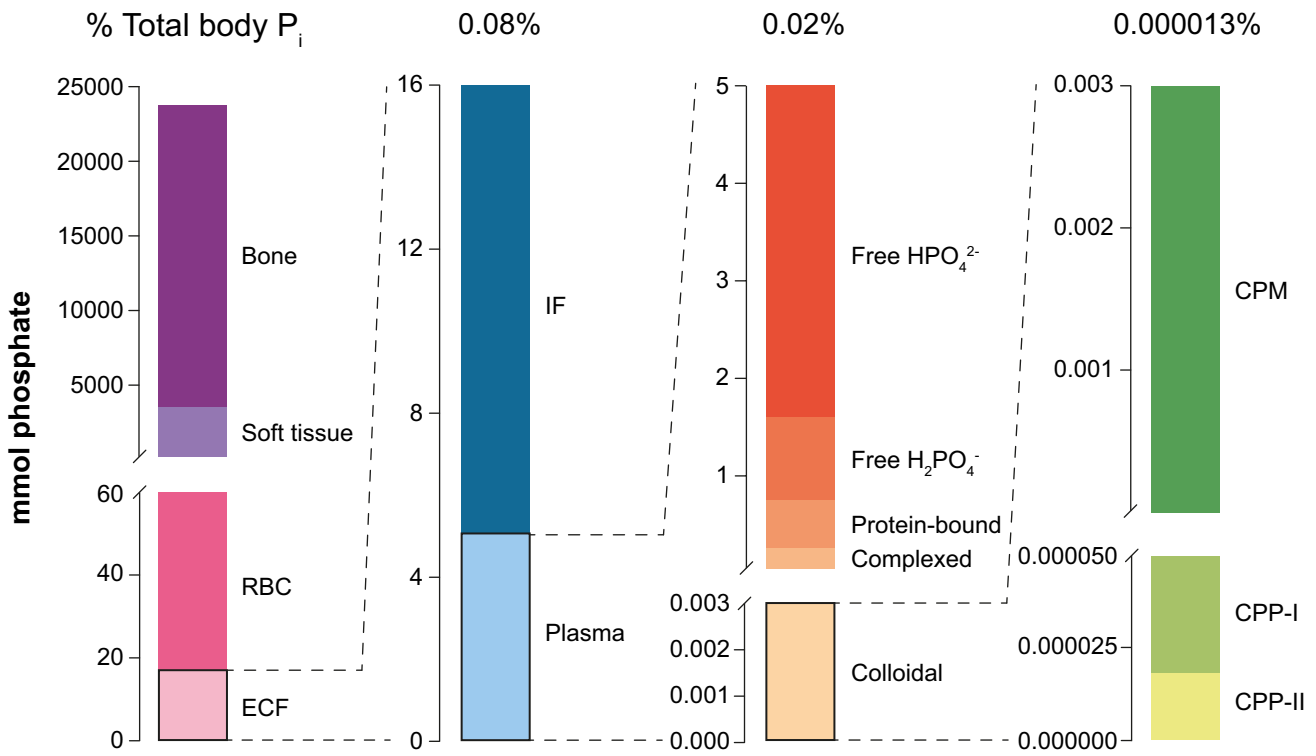

Fig. 2 Negligible contribution of circulating colloidal (calcium) phosphate to total body and plasma inorganic phosphate. Approximate distribution of inorganic phosphate (24,000 mmol) across body compartments in a 75-kg adult. Assumptions: IF constitutes ~ 75% of ECF volume. IF phosphate concentration is ~ 85% of plasma [91]. CPM, CPP-I, and CPP-II collectively comprise the major colloidal fraction of (calcium) phosphate carriage in plasma. The majority (95%) of phosphate resides with CPM. CPP-I typically outnumber CPP-II by 10:1. CPM (equivalent to 5 nM fetuin-A, each molecule bound to 100 Posner-type clusters), CPP-I (10^6/mL), and CPP-II (10^5/mL) phosphate content is estimated empirically as per text. Other phosphate data was taken from [91]. ECF, extracellular fluid; IF, interstitial fluid; CPM, calciprotein monomer; CPP, calciprotein particle; RBC, red blood cells

further reveal detrimental effects on the vasculature, including a dose-dependent loss of endothelium-dependent vasorelaxation [31], heightened arterial stiffness [88], and lesional particle uptake in atherosclerosis models [49]. Although some evidence suggests more pronounced effects with CPP-I, attributing toxicity to these earlier stage particles is complicated by their propensity to transform over the course of experimentation and further work is needed to clarify these findings.

Their inherent instability also complicates in vivo investigations. The tendency of CPP to aggregate or transition into different states precludes the use of chronic delivery systems or extended administration regimens, so existing data remain limited and treatment timeframes necessarily short. In mice given a single intravenous (IV) bolus of CPP-II, Anzai et al. documented IL-1β– and IL-1α–dependent inflammatory responses and pronounced neutrophil influx when administered intraperitoneally [5]. Whereas Neutel et al. observed no vascular stiffening after repeated IV injections (thrice weekly for 2 weeks) in wild-type mice [88], Shiskova and colleagues reported molecular changes suggestive of endothelial activation and defective mechanotransduction in rats injected IV daily for 10 days [116]. The same group found that single or thrice-weekly injections for 5 weeks produced intimal hyperplasia and adventitial/perivascular inflammation in rats bearing balloon catheter–injured aortas and, remarkably, even in animals with uninjured aortas [118].

These observations mirror many features of phosphate-induced toxicity [62]. Nevertheless, it must be recognized that nearly all such mechanistic insights originate from *synthetic* CPP preparations that differ both quantitatively and qualitatively from physiologically occurring forms. In every instance, these CPP were generated by supersaturating solutions of Ca and Pi in the presence of either bovine fetuin-A (BF) as the sole protein source or foetal calf serum (FCS), which is rich in fetuin-A [150]. Prolonged incubations of 12 h to 14 days typically yield large, minimally substituted, highly crystalline hydroxyapatite–laden CPP (CPP-II) that are rarely seen in normal plasma. By contrast, endogenous CPP are generally smaller, relatively mineral poor, and predominantly composed of ACP. If any crystalline component is present, it is typically poorly crystalline, non-stoichiometric hydroxyapatite substituted with sodium, magnesium,

and carbonate [127, 150]. Although the terms "CPP-I" and "CPP-II" offer a useful conceptual framework, this classification is overly simplistic. The transition from CPP-I to CPP-II is not abrupt; cryo-TEM reveals "intermediate" forms that contain both amorphous and crystalline features [127, 128]. In nano-flow cytometry studies, these nascent crystal domains are identified by their enhanced bisphosphonate binding. Consequently, "CPP-II" generally denotes particles with some surface-oriented crystal structures, even though they may still harbor primarily amorphous mineral. Neither BF- nor FCS-derived CPP display the full biomolecular corona characteristic of native human CPP, a corona likely to change with feeding status and disease context [127]. Moreover, depending on the specific protocol, these supersaturated conditions yield a vast number of particles, around 10^{11} to 10^{15} per milliliter, capturing up to half of the total mineral ions in colloidal form [47]. The resulting CPP are pelleted, washed, and used directly in subsequent experiments.

Next arises the issue of dosing and measurement. Most investigators define the CPP dose by colorimetrically measured calcium content (after dissolving samples in acid), or simply the number of reaction tubes (pellets) used. Surveying the literature, typical dosing regimens involve applying 20–600 µg calcium/mL (i.e., 0.5–15 mM), which far surpasses endogenous CPP levels (in the nM range) and even exceeds total plasma calcium in some instances. Such approaches may significantly inflate the apparent toxicity. In static, closed, in vitro systems, cells literally become engorged on particles, which is unlikely to be representative of any phenomenon that occurs in vivo [97].

To better understand CPP-induced dysfunction, experiments should approximate in vivo scenarios using realistic particle concentrations, synthesized under more representative conditions, and considering flow dynamics that mimic circulation. Such refinements will help distinguish actual pathophysiological roles from artefacts. Longitudinal, low-level exposure models using organ-on-a-chip systems may reveal cumulative impacts that short-term, high-dose cell culture experiments miss.

Nonetheless, while the relevance of these findings using very high-dose CPP is debatable, prolonged exposure to marginally elevated CPP levels or more labile CPM/CPP mixtures may contribute subtly to chronic vascular stress and inflammation. Indeed, a recent transcriptomic analysis of arterial biopsies obtained at surgery revealed a strong association between circulating CPP levels and enrichment for markers of endothelial activation, inflammation, and ECM remodeling [32]. Over many years, this cumulative burden could help explain the strong associations between higher CPP levels, particularly CPP-II, and mortality risk in CKD patients [138]. These findings build on a growing body of observational data in CKD cohorts, where circulating CPP levels have been linked to markers of inflammation [121, 123, 124, 126, 128, 134], oxidative stress [124], and, in some studies, arterial stiffness [124], although not consistently [140]. Moreover, removing the CPP-containing fraction from uremic serum significantly reduces its capacity to induce endothelial activation, inflammation, and VSMC mineralization in vitro [134].

Perhaps the most compelling direct evidence of CPP pathogenicity comes from a recent study by Miura et al. [77], where nephrectomized miniature pigs were treated with a modified hemodialysis circuit incorporating an alendronate-based CPP adsorption column. In that setting, removing CPP from circulation improved survival and alleviated coronary artery calcification, vascular endothelial dysfunction, metastatic pulmonary calcification, left ventricular hypertrophy, and chronic inflammation. Crucially, these effects occurred without altering total serum phosphate, underscoring that CPP, especially CPP-II, which are preferentially bound by the column resin [78] rather than free phosphate ions, played the key injurious role.

In contrast to CPP, relatively little is known about the bioactivity of CPM. The limited available data suggest that CPM are relatively inert: one study, for instance, found no detectable cytotoxic effects on cultured proximal tubule cells, macrophages, or LSECs despite high loading (2.5 mM calcium) [60]. Furthermore, CPM do not appear to activate the NLRP3 inflammasome [60]. Whether their accumulation in tissue fluid could foster a pro-calcific environment remains unclear. Still, CPM evidently harbor the potential to aggregate into larger, potentially more inflammatory species if conditions permit.

The pathogenic potential of CPM aggregates within the kidney, in particular, warrants further investigation. Notably, blocking fetuin-A/CPM uptake in rats mitigates PTH-induced nephrocalcinosis [71], suggesting that preserving fetuin-A levels in the tubular fluid may be protective. Indeed, lower urinary fetuin-A concentrations have been associated with a higher risk of stone formation [74]. Conversely, in the absence of mechanisms to maintain adequate tubular fetuin-A levels, high dietary phosphate loading in mice promotes intraluminal calcium phosphate microcrystal formation, which in turn drives tubular damage, interstitial fibrosis, calcification, and progressive nephron loss [113].

Reassessing the relevance of CPP to vascular calcification

An intuitive model that has CPP depositing in vessel walls and serving as a nidus for calcification, falters upon closer examination. Anatomical barriers, rapid clearance, and the sheer rarity of CPP in circulation make consequential direct seeding improbable. Notably, even after 72 h of exposure at concentrations 100-fold higher than typical endogenous

levels, Zeper et al. reported that native CPP isolated from dialysis patients induced only a modest increase in VSMC mineralization compared to vehicle controls [150]. This effect appears minor relative to the robust calcification observed with high phosphate or other pro-calcific stimuli. Particles with CPP-II-like morphology are also conspicuously absent from the many detailed ultrastructural studies on calcifying arteries [98, 110, 144]. Moreover, several cross-sectional analyses report null associations between circulating CPP levels and vascular calcification scores [32, 140]. Instead, CPP's pathological influence on vascular calcification (if any) likely stems from more indirect mechanisms like promoting endothelial dysfunction, fuelling low-grade inflammation, and influencing macrophage/immune cell phenotypes. These subtle changes accumulate, setting the stage for calcification triggered by other stimuli. Indeed, if not cleared, the cell types circulating CPP are most likely to encounter are blood cells, where a preference for monocytes has been observed [115], and endothelial cells.

For example, if, as suggested by in vitro and ex vivo data, CPP indirectly induces phenotypic changes in the endothelium, such as reducing nitric oxide bioavailability [31], increasing oxidative stress [31, 114], endothelial-to-mesenchymal transition [116], and elevating the expression of adhesion molecules [115, 116, 134], then they could well prime the vascular wall for maladaptive remodeling, infiltration of monocytes, and local inflammation, all processes that may predispose to atheroma and vascular calcification over long timeframes [30, 112]. Rather than CPP acting as the direct initiators of calcification at the tissue level, their role may lie in setting the stage for other pathological processes, shifting the balance toward a state more conducive to calcification mediated by local cells and extracellular matrix components.

Separating CPPs, T50, and vascular calcification

Several research-based assays are currently employed to measure CPM and CPP in biological fluids, each founded on different analytical principles [120]. Among these, the nano-flow cytometry (nano-FC) represents the most sophisticated approach. In this method, CPP is labeled using either a fetuin-A tracer or mineral-binding dyes, while vesicular particles (e.g., extracellular vesicles) are excluded using membrane-specific dyes [42, 126, 128]. Uniquely, nano-FC can distinguish CPP-I from CPP-II by exploiting their differential affinity for bisphosphonates—newly formed crystalline surfaces bind bisphosphonates more strongly—or through variations in light scatter (which reflect differences in size and refractive index). Only nano-FC has the distinct capability to quantify particle numbers and classify particle subtypes. Published data support its analytical performance, demonstrating acceptable precision, accuracy, and sample stability [126, 128].

A second approach, known as the "gel filtration" method, also utilizes fluorescent bisphosphonate to detect mineral phases [78]. In this process, denser CPP can be pelleted by centrifugation, leaving predominantly CPM (or "low-density" particles) in the supernatant. The sample is then incubated under conditions that encourage CPM aggregation and transformation into CPP-II, thus enabling their subsequent detection with Osteosense [79]. However, the term "gel filtration" is somewhat misleading, as it primarily serves to remove excess dye rather than to separate CPM from CPP.

Earlier methods based on fractional immunoassay of fetuin-A across various density fractions [41] are less specific for CPM/CPP and have largely been replaced by these newer assays [119]. None of the current methods are standardized or fully calibrated; however, a recent side-by-side comparison demonstrated unexpectedly good agreement among the different techniques [138]. Notably, only the nano-FC-based measurement of CPP-II was independently associated with mortality and CKD progression. Although none of these methodologies are validated for clinical use, they have been extensively applied in clinical studies: nano-FC [9, 16, 32, 103, 118, 126, 128, 134–138, 141], gel filtration [35, 43, 78, 80, 83, 84, 89, 136–138], and fetuin-A reduction ratio [14, 41, 121, 123, 124], providing broadly consistent insights into the potential pathophysiological relevance of these mineral-protein complexes.

In contrast to these assays of endogenous CPM/CPP, the "T50 test" evaluates a serum's capacity to prevent *new* crystal formation when supersaturated with calcium and phosphate under controlled temperature and pH [92]. In bulk solution, the conversion from CPP-I to CPP-II leads to increased light scattering, which can be monitored in real-time through turbidimetry or nephelometry, providing a time-to-half-maximum crystallisation (T50) [94]. This metric encompasses the activity of various inhibitory and promoting factors but operates under conditions fundamentally different from in vivo environments.

These nuances become more apparent when considering that much of the nascent CPP pool likely arises outside the intravascular compartment, such as in bone remodeling microenvironments or within the gut, where ionic and proteomic conditions may deviate substantially from what the T50 test probes. Moreover, T50 captures crystallisation over several hours [94], whereas CPM/CPP often dissipate within minutes in vivo [60, 63], aided by rapid clearance mechanisms and local physiologic variations not mirrored in the closed T50 system. Thus, it simulates a "stress test" for serum but does not replicate in vivo kinetics of formation, clearance, or compartmental origin of CPM/CPP. Indeed, some patients can exhibit comparatively "normal" T50 readings yet harbor high baseline CPP, possibly

reflecting frequent microenvironmental spikes that never fully translate into serum crystallization under forced conditions. Conversely, others might show short T50 times but maintain low-circulating CPP counts, implying a theoretical susceptibility to crystallization that is seldom realized in vivo thanks to swift clearance or the absence of sustained oversaturation challenges. In other words, a shortened T50 reflects a serum environment favouring faster crystal growth under artificial conditions, but it may not correlate with higher steady-state CPM/CPP levels in circulation. There is evidence in the literature supporting this dissociation. For instance, the postprandial rise in CPM and CPP is *not* accompanied by the anticipated drop in T50 [136]; instead, T50 increases slightly, likely reflecting a modest rise in fetuin-A. Another example can be seen in dialysis patients treated with etelcalcetide [135], where T50 remained unchanged despite a marked reduction in CPM and CPP. Consequently, gut- or bone-driven interventions that substantially alter CPP biology in vivo do not necessarily translate into a corresponding change in T50 ex vivo.

Although often conflated, T50 and direct CPP measurements are neither equivalent nor interchangeable. Critically, the T50 test does not quantify the abundance or types of native CPP, and removing endogenous CPP from serum does not affect the resulting T50 value [122]. Thus, combining T50, which indicates how quickly serum might crystallize under stress, with direct CPM/CPP quantification which reflects the day-to-day colloid burden, provides a more comprehensive perspective.

Interpreting T50 solely as a "calcification propensity" oversimplifies the underlying biology. A lower T50 does not necessarily lead to overt vascular calcification—indeed, the data are equivocal [13, 21]. Nevertheless, it may still indicate an overall susceptibility that, in combination with local microenvironmental factors, predisposes certain patients, particularly those with CKD, to vascular complications over time. This process may or may not involve mineral deposition; hence, neither T50 nor CPP should be viewed as direct biomarkers of vascular calcification. How, then, can we reconcile the consistent association between lower T50 values and poorer outcomes across multiple cohorts? One plausible mechanism is that a shorter T50 may reflect conditions favoring advanced CPP-II formation. Although recent findings correlate elevated CPP-II with increased mortality in CKD [138], it remains uncertain whether CPP-II alone fully explains these outcomes. If T50 serves not merely as an aggregate of various individual risk factors (e.g., phosphate, magnesium) but also as an index of accelerated CPP-II maturation, then its link to pathology appears logical.

The modified particle concept—a unifying hypothesis?

Emerging evidence raises the prospect that surface "decorations" or adsorbed ligands may be more critical to CPP pathogenicity than their mineral cores [127, 134]. The biomolecular corona surrounding CPP is not simply an arbitrary accumulation of trace contaminants; many of these molecules bind with sufficient specificity or affinity to confer distinct bioactivities. Certain ligands, from complement factors and acute-phase reactants to cytokines/growth factors, extracellular matrix components and microbial remnants, may significantly influence how CPP interact with cells and tissues. Importantly, the more crystalline surfaces of CPP-II can substantially enhance ligand-binding capacity. Indeed, HAP is widely employed in industry for toxin removal and microextraction, precisely because of its vast specific surface (up to ~ 250 m^2/g) for adsorption and exchange [81]. Accordingly, modified or "ligand-laden" CPP-II may assume inflammatory or immunogenic features not present in simpler, amorphous species. A parallel exists with modified LDL in atherogenesis, where modifications to the particle, rather than the lipid content per se, are considered more decisive drivers of immune and inflammatory processes [130].

Speculatively, the site at which CPP arise may also shape the composition of their biomolecular corona and, hence, their pathogenic attributes. For instance, gut-derived CPP, formed in a microbe-rich intestinal milieu, may more readily integrate endotoxin-like ligands, heightening pro-inflammatory or immunostimulatory effects. By contrast, CPP generated in bone remodeling regions might incorporate fewer microbial signals but may adsorb osteogenic or matrix-associated molecules. Characterization of CPP-II in ESKD highlights significant alterations in the corona such as apolipoprotein content (ApoA1, ApoA4, ApoC3) is elevated protein-bound uraemic toxins (e.g., indoxyl sulfate, p-cresyl sulfate); lipids (particularly saturated fatty acids and n6-PUFAs) are more abundant, growth factors, especially from the transforming growth factor-beta superfamily, become enriched; and microbial products such as endotoxin, bacterial DNA, and peptidoglycan have thus far been detected only in ESKD-derived CPP-II, but not in healthy forms [127]. Importantly, recent studies indicate that CPP from CKD patients exhibit significantly greater pro-calcific and pro-inflammatory activity than their non-uraemic counterparts [134, 150]. Crucially, at equivalent particle concentrations (~ 10^8/mL, about 100-fold above endogenous levels), uremic CPP induces greater IL-1β, IL-6, and IL-8 release in endothelial cell cultures and *uniquely* stimulate TNFα, IL-1α, IL-18, and IL-23 secretion [134]. These observations underscore how a pathological milieu can reshape the corona,

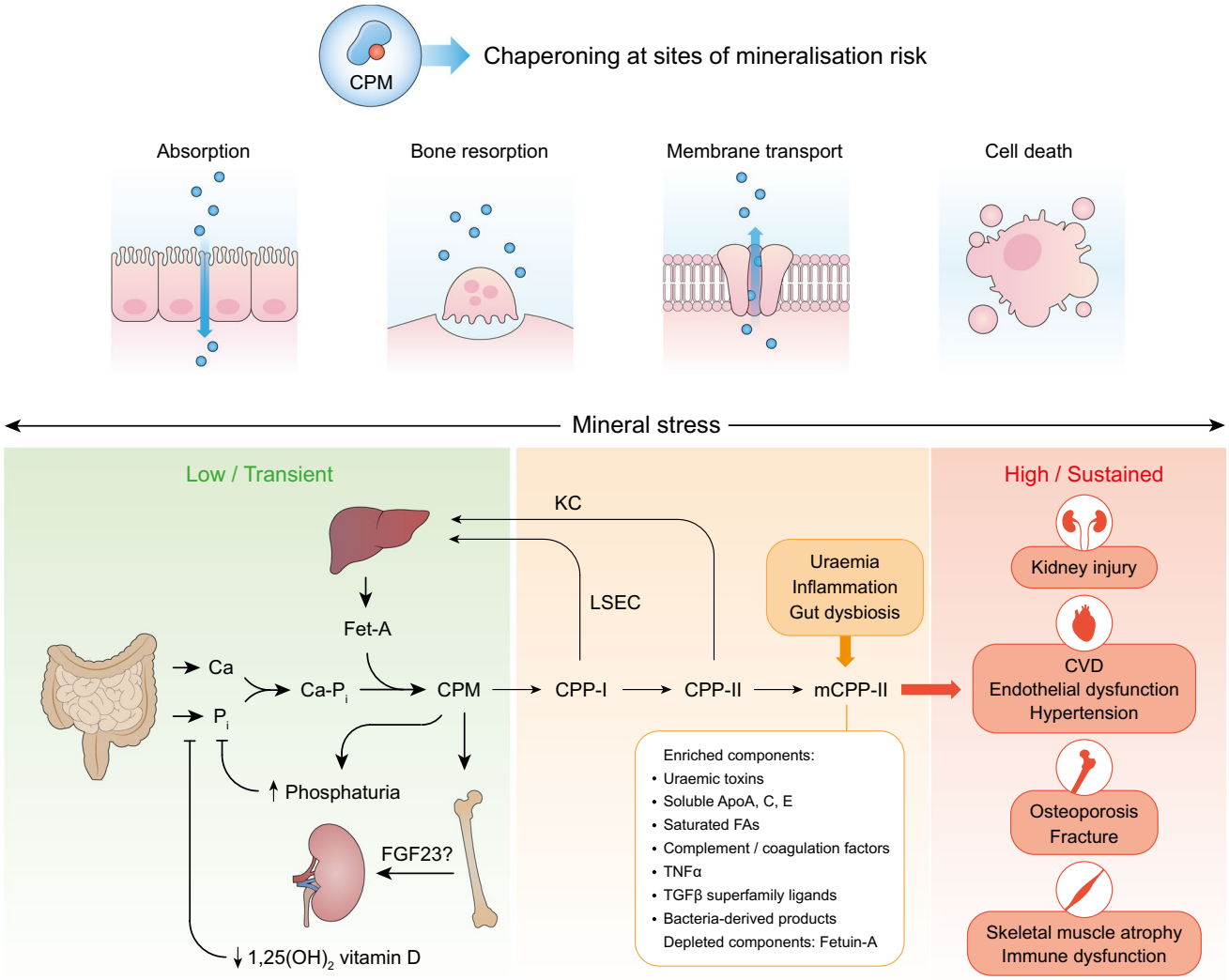

Fig. 3 Hypothetical pathways of CPM/CPP metabolism in health and disease. Depicted here are the hypothetical physiological and pathological pathways centred on calciprotein monomers (CPM) as the primary buffers of transient mineral surges. In normal physiology (green), dietary calcium and phosphate (Ca–P_i) entering from the intestine may transiently exceed local solubility thresholds. CPM swiftly form through the binding of nascent ion clusters by fetuin-A (Fet-A) and possibly other proteins, acting as molecular chaperones in the gut lumen, bone remodeling surfaces, and other sites of high ion flux or cell turnover. These short-lived CPM help prevent inadvertent crystallization, buffering epithelial and bone microenvironments. Emerging data also suggest CPM might deliver mineral or signals that prompt endocrine regulation via fibroblast growth factor 23 (FGF23) secretion from bone, thereby influencing renal phosphate excretion and systemic Ca–P_i homeostasis. In states of sustained mineral stress (orange- > red), CPM may aggregate to form primary calciprotein particles (CPP-I) and, ultimately, crystalline secondary CPP (CPP-II). Although many of these are rapidly cleared by liver sinusoidal endothelial cells (LSEC) or Kupffer cells (KC), prolonged mineral stress coupled with gut dysbiosis, inflammation, or reduced Fet-A/magnesium/pyrophosphate can yield persistently modified CPP-II (mCPP-II). These advanced particles are enriched with apolipoproteins, saturated lipids, uremic toxins, and microbial products—contributing to renal, cardiovascular, bone, and other aging-related sequelae. The net outcome reflects a balance between CPM's protective, rapid buffering role, and the potential pathological cascade triggered by persistent, ligand-laden mCPP-II

potentially magnifying or conferring new pro-inflammatory or immune-reactive properties. Thus, even at the low levels observed in vivo, CPP have the potential to initiate or amplify cellular signalling cascades and modify cell phenotypes without requiring overt calcification. Indeed, in the context of CKD, mCPP-II might be considered uremic toxins.

Knowledge gaps

Substantial uncertainties remain. We have limited clarity on the frequency and composition of CPP in normal physiology compared to disease states. The relative contributions of gut- and bone-derived CPM/CPP to the plasma pool are not fully delineated. Nor do we precisely know how

repeated small pulses of CPM might integrate with or subtly augment phosphate homeostatic signals. While in vitro data suggest that modified or "decorated" CPP-II might contribute to vascular pathology, it remains unclear whether specifically targeting the amorphous-to-crystalline conversion or removing advanced CPP could substantively attenuate CKD-related vascular damage. Lastly, exploring other putative chaperones beyond fetuin-A, particularly in compartments with little fetuin-A, could reveal deeper evolutionary redundancies in this protective system.

Summary

CPM/CPP do not transport large fractions of the body's Ca–P_i load at any one time, but enact critical short-term and localized buffering, bridging the gap between the near-instant nucleation risk and slower hormone-mediated controls. Evolving under the imperative to preserve skeletal mineralization while thwarting soft-tissue calcification, they help keep vertebrate plasma in a metastable state. Figure 3 provides a hypothetical framework for understanding the role of CPM/CPP in physiology and disease. Microenvironments like the gut and bone surfaces spawn these colloids, while the kidneys and liver usually remove them before they fully crystallize or accumulate injurious ligands. In CKD and other conditions of disturbed mineral metabolism, the equilibrium upholds a subtle but increasingly pertinent role for CPM/CPP, facilitating or foiling pathologies via their dynamic interplay with other inhibitors and clearance pathways. Future advances must illuminate which exact forms and ligands might push them from ephemeral protectors to pathogenic factors, and how best to harness or restrict these transformations for potential clinical benefit.

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Author contributions E.R.S. planned, conceptualised and wrote the initial draft. S.G.H. revised the manuscript. Both authors proofread and approved the final version of the manuscript.

Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests E.R.S. has received speaker honoraria from CSL Vifor and owns stock in Calcison AG. S.G.H. has received speaker honoraria from CSL Vifor, Baxter, AstraZeneca.

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