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Kutikhin, Anton G; Feenstra, Lian; Kostyunin, Alexander E; Yuzhalin, Arseniy E; Hillebrands, Jan-Luuk; Krenning, Guido

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BRIEF REVIEW

Calciprotein Particles

Balancing Mineral Homeostasis and Vascular Pathology

Anton G. Kutikhin,* Lian Feenstra,* Alexander E. Kostyunin, Arseniy E. Yuzhalin, Jan-Luuk Hillebrands, Guido Krenning

ABSTRACT: Hypercalcemia and hyperphosphatemia associate with an elevated risk of cardiovascular events, yet the pathophysiological basis of this association is unclear. Disturbed mineral homeostasis and the associated hypercalcemia and hyperphosphatemia may result in the formation of circulating calciprotein particles (CPPs) that aggregate the excessive calcium and phosphate ions. If not counteracted, the initially formed harmless amorphous spherical complexes (primary CPPs) may mature into damaging crystalline complexes (secondary CPPs). Secondary CPPs are internalized by vascular cells, causing a massive influx of calcium ions into the cytosol, leading to a proinflammatory response, cellular dysfunction, and cell death. Although the pathophysiological effects induced by CPPs in vascular cells receive increasing attention, a complete picture of how these particles contribute to the development of atherosclerosis and vascular calcification remains elusive. We here discuss existing knowledge on CPP formation and function in atherosclerosis and vascular calcification, techniques for investigating CPPs, and models currently applied to assess CPP-induced cardiovascular pathogenesis. Lastly, we evaluate the potential diagnostic value of serum CPP measurements and the therapeutic potential of anti-CPP therapies currently under development.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: atherosclerosis ■ calcium ■ homeostasis ■ hypercalcemia ■ hyperphosphatemia ■ vascular calcification

Calciprotein particles (CPPs) are blood-borne circulating particles formed of a combination of calcium phosphate and protein.[1,2] Their clinical importance stems from the observation that circulating CPP levels are elevated in patients with chronic kidney disease[3,4] where vascular calcification develops earlier compared to healthy subjects.[5,6] Indeed, increased circulating CPP levels associate with arterial stiffness[4] and the development and progression of calcific uremic arteriopathy,[3] atherosclerosis,[7] and vascular calcification.[8] Moreover, the propensity of serum to form CPPs is associated with the occurrence of cardiovascular events and mortality.[9–15] Albeit the pathophysiological effects of CPPs receive increasing attention, mechanistic insight into how these particles contribute to the development of atherosclerosis and vascular calcification remains elusive. In this review, we discuss existing knowledge on CPP formation and function in atherosclerosis and vascular calcification, the techniques to investigate CPPs, and models currently applied to assess CPP-induced cardiovascular pathogenesis.

CALCIUM AND PHOSPHATE HOMEOSTASIS AND THE GENERATION OF CPPS

Serum calcium and phosphate levels are tightly regulated in the human body. Calcium and phosphate metabolism includes their intestinal absorption, deposition and resorption from the bone, and renal reabsorption, regulated by

Correspondence to: Anton G. Kutikhin, PhD, Laboratory for Vascular Biology, Division of Experimental and Clinical Cardiology, Research Institute for Complex Issues of Cardiovascular Diseases, 6 Sosnovy Blvd, Kemerovo, 650002, Russian Federation, Email antonkutikhin@gmail.com; or Guido Krenning, PhD, Laboratory for Cardiovascular Regenerative Medicine, Dept. Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Hanzeplein 1 (EA11), 9713GZ Groningen, the Netherlands, Email g.krenning@umcg.nl

*A.G. Kutikhin and L. Feenstra contributed equally.

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Nonstandard Abbreviations and Acronyms

BMP	bone morphogenic protein
Ca^{2+}	ionized calcium
CaSR	calcium-sensing receptor
CKD	chronic kidney disease
CMVs	calcifying microvesicles
CPPs	calciprotein particles
ECs	endothelial cells
eNOS	endothelial nitric oxide synthase
ESRD	end-stage renal disease
GRP	γ-carboxylated glutamate–rich protein
HAP	hydroxyapatite
IL	interleukin
MGP	matrix γ-carboxylated glutamate protein
MSR	macrophage scavenge receptor
MSX	homeobox transcription factor muscle segment homeobox
NF-κB	nuclear factor kappa B
RANKL	receptor activator of nuclear factor κB ligand
RUNX	runt-related transcription factor
SOX	sex-determining region Y-box
TACT	trial to assess chelation therapy
TLR	toll-like receptor
TNF	tumor necrosis factor
VSMCs	vascular smooth muscle cells

Highlights

- This review discusses the contribution of calciprotein particles to the pathogenesis of atherosclerosis and vascular calcifications. The important determinants of calciprotein particle formation and the pathogenic processes wherein calciprotein particles are involved are highlighted.
- Calciprotein particles are internalized by vascular cells, causing a massive influx of calcium ions into the cytosol, leading to a proinflammatory response, cellular dysfunction, and cell death.
- Calciprotein particles are a modifiable risk factor for the development of cardiovascular events.
- Pioneering anti-calciprotein particle therapies reduce the risk of cardiovascular events.

calciotropic and phosphotropic factors (reviewed in Renkema et al,[16] Peacock,[17] Peacock,[18] Blaine et al[19]). Mechanisms maintaining calcium and phosphate homeostasis are redundant and interconnected,[16] and their dysregulation may result in hypercalcemia and hyperphosphatemia as well as extraskeletal calcifications, including vascular calcifications.[17,18]

A network of endogenous inhibitors, with distinct mechanisms of action, prevents and inhibits the formation of extraskeletal calcifications.[20] First, the prevention of bone resorption, the decrease in calcium and phosphate reabsorption by the kidneys, and the inhibition of calcium phosphate crystal growth all inhibit extraskeletal calcification. Osteoprotegerin is a decoy RANKL (receptor for the receptor activator of NFκB [nuclear factor κB] ligand)[21] precluding osteoclastic differentiation, activation, and bone resorption.[22,23] Osteopontin inhibits osteoclastic differentiation and bone resorption, but its vascular expression promotes mineral resorption via unknown mechanisms.[24–26] Klotho is a coreceptor for fibroblast growth factor 23 that abates phosphate reabsorption in kidney proximal tubules and biosynthesis of calcitriol, thereby reducing renal tubular calcium reabsorption and intestinal calcium and phosphate absorption.[27] Furthermore, inorganic pyrophosphate hinders the nucleation and crystallization of amorphous calcium and inhibits the growth of mature hydroxyapatite crystals.[20]

Second, circulating calcium scavengers buffer the amount of free calcium available for extraskeletal calcification. Albumin binds ionized calcium (Ca^{2+}) via its negatively charged amino acids distributed on the surface of the tertiary protein structure, scavenging Ca^{2+} from the microenvironment.[1] Similarly, osteonectin scavenges Ca^{2+} via multiple negatively charged amino acids focused on specific domains, for example, EF-hand (helix-loop-helix) domain.[28]

Third, CPPs scavenge both free Ca^{2+} and phosphate (PO_4^{3-}) ions and sequestering minerals available for extraskeletal calcification. CPPs are blood-borne spongeous carbonate-hydroxyapatite particles, 50 to 500 nm in diameter[29,30] that adsorb proteins from their environment.[31,32] Fetuin-A, MGP (matrix γ-carboxylated glutamate protein) and GRP (γ-carboxylated glutamate–rich protein) scavenge Ca^{2+} and PO_4^{3-} ions from the serum and complex these into clusters of protein and amorphous calcium phosphate ($Ca_3[PO_4]_2$).[1,2,33,34] Fetuin-A scavenges serum Ca^{2+} and PO_4^{3-} via its negatively charged extended β-sheet within the amino-terminal cystatin-like D1 domain[1,33] and stabilizes nascent clusters of calcium phosphate in its monomeric form[33] (Figure 1A). MGP and GRP contain negatively charged γ-carboxylated glutamate residues[34,35] which bind both Ca^{2+} and calcium-containing compounds (Figure 1A).[36–39] The interaction between fetuin-A and MGP integrates calcium and phosphate clusters into amorphous proteinaceous spherical particles called primary CPPs (Figure 1B). In physiology, these initially formed primary CPPs are generally considered harmless and facilitate clearance of calcium and phosphate. However, in conditions of hypercalcemia or hyperphosphatemia, primary CPPs ripe into harmful needle-shaped crystalline secondary CPPs containing calcium hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$) by a process called amorphous-to-crystalline transition[31,40]

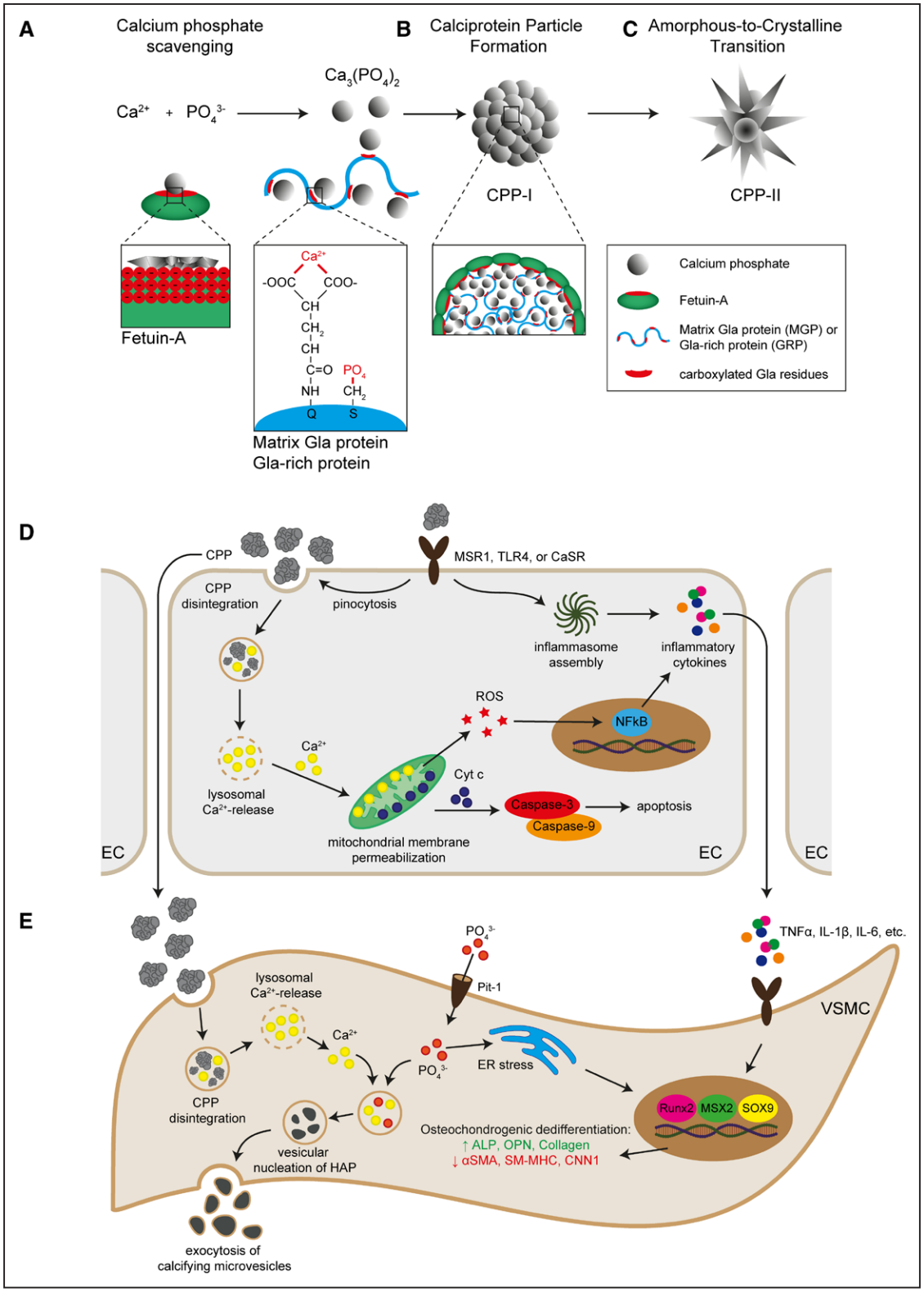

Figure 1. Calciprotein particle (CPP) formation and pathophysiological mechanisms.
In the blood, Ca^{2+} and $PO4^{3-}$ form complexes of calcium phosphate that can be scavenged by fetuin-A via the β-sheet of the amino-terminal cystatin-like D1 domain, which contains multiple negatively charged amino acids. MGP (matrix γ-carboxylated glutamate protein) and GRP (γ-carboxylated glutamate–rich protein) scavenge calcium phosphate via their negatively charged amino acids in the γ-carboxylated glutamate residues. Additionally, MGP and GRP scavenge $PO4^{3-}$ via the phosphorylation of serine residues (**A**). The interaction between fetuin-A and MGP integrates calcium phosphate into amorphous spherical particles named primary CPP (**B**). These primary CPP may ripe into highly crystalline CPP (secondary CPP) under conditions of hypercalcemia and hyperphosphatemia (**C**). (*Continued*)

(Figure 1C). Serum fetuin-A levels inversely associate with secondary CPP formation,[13,41] implying that fetuin-A may act as an inhibitor of amorphous-to-crystalline transition.[31] The key determinants of amorphous-to-crystalline transition need further investigation.

MGP, GRP, and fetuin-A are essential to calcium and phosphate homeostasis as mice lacking either protein spontaneously develop extraskeletal calcifications in soft tissues. MGP- and GRP-deficient mice develop medial arterial calcifications[34,42,43] and may prematurely die from blood vessel rupture.[34] Fetuin-A–deficient mice develop numerous calcified thrombi in the microvasculature[44,45] and intimal arterial calcifications on atherosclerosis-prone genetic backgrounds.[46] Exogenous fetuin-A supplementation inhibits the development of calcified thrombi in fetuin-A–deficient mice, confirming its relevance to vasculopathy.[44] Expectedly, serum Ca^{2+}, PO_4^{3-}, low fetuin-A, and high CPP levels all associate with the development of vascular pathology.[47–49]

Hereinafter, it must be noted that proteinaceous CPPs should be clearly distinguished from inorganic calcium phosphate crystals, although an identical mineral composition of these entities may evoke similar downstream events.

CPPS IN CARDIOVASCULAR PATHOPHYSIOLOGY

Internalization, Cell Death and Proinflammatory Signaling

CPPs exert considerable cytotoxic effects on multiple vascular and valvular cell types, including vascular endothelial cells (ECs),[32] vascular smooth muscle cells (VSMCs),[50] adventitial fibroblasts,[51] valve interstitial cells, and valvular ECs.[52]

Internalization of CPPs is an active process that may occur via clathrin-mediated endocytosis, involving MSR (macrophage scavenge receptor) 1 scavenger receptors and actin polymerization[53–55](Figure 1D). CPP shape and crystallinity greatly impact internalization,[54] and different cell types have distinct internalization efficacies. Macrophages preferentially internalize secondary CPPs, whereas ECs preferentially internalize primary CPPs.[54] The molecular basis behind these distinct internalization patterns is currently unknown but may reflect distinct receptors for primary and secondary CPPs. Indeed, knockdown of the *MSR1* gene or blockade of the MSR1 receptor in macrophages diminishes the internalization of secondary CPPs without affecting the internalization of primary CPPs.[53,54] Furthermore, the CaSR (calcium-sensing receptor) is expressed on a variety of vascular cells, including ECs, smooth muscle cells, and monocytes[56,57] and offers an alternative route for CPP internalization. Blood monocytes internalize secondary CPPs via the CaSR in a Ca^{2+} concentration-dependent manner, but independently of PO_4^{3-}.[56] Of note, the internalization of inorganic calcium phosphate crystals is also accomplished by clatherin-mediated endocytosis and macropinocytosis,[58] suggesting that CPPs and calcium phosphate crystals use similar internalization routes (Figure 1D).

Cytochalasin D, chlorpromazine, and polyinosinic acid lower CPP internalization rates regardless of their physical or chemical properties, indicating that although different surface receptors are responsible for the CPP binding, the downstream mechanism of internalization is similar.[53,54] Nevertheless, it should be noted that the mechanisms of CPP internalization have received limited attention to date and need further investigation and independent confirmation.

Inorganic calcium phosphate crystals induce cell death via Ca^{2+}-dependent mitochondrial outer membrane permeabilization.[59] Controversy exists as to the exact mechanism of the cytosolic calcium influx; some experimental results indicate mild lysosome membrane permeabilization[59,60]; other studies report severe lysosomal rupture due to the osmotic difference between the crystal-carrying lysosomes and the cytosol.[61] CPPs also induce cell death in a variety of vascular cells, albeit to a lesser extent,[32,62,63] and it is tempting to speculate that CPP-induced cell death occurs via similar mechanisms. Of note, the incorporation of fetuin-A into calcium phosphate crystals—effectively generating secondary CPPs—dose-dependently decreases cytotoxicity by limiting particle-induced intracellular Ca^{2+} elevations.[63] The exact mechanism by which CPPs induce cell death remains unclear and may differ between primary and secondary CPPs, as these have distinct crystallinity and therefore solubility in lysosomes.[54] Nonetheless, cleavage of caspase-3 and caspase-9 following CPP internalization by vascular cells implies a central role for intrinsic apoptosis (Figure 1D).[32,64]

CPPs induce expression and secretion of proinflammatory cytokines, including IL (interleukin)-1β, IL-6, IL-8,

Figure 1 Continued. Endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) can internalize CPP via receptor-mediated pinocytosis. In ECs, CPP internalization induces a rise in intracellular Ca^{2+} level, which results in the inflammatory activation of the ECs, characterized by increased transcellular permeability, oxidative stress, and inflammatory cytokine production (**D**). In VSMCs, CPP internalization results in a rise in intracellular Ca^{2+} and $PO4^{3-}$ levels that evoke osteochondrogenic dedifferentiation via various mechanisms including inflammatory signaling and oxidative stress. An important molecular consequence of osteochondrogenic dedifferentiation of VSMCs is the production and excretion of calcifying microvesicles, which facilitate vascular calcification (**E**). α-SMA indicates alpha smooth muscle actin; ALP, alkaline phosphatase; CaSR, calcium-sensing receptor; CNN, calponin; ER, endoplasmatic reticulum; HAP, hydroxyapatite; IL, interleukin; MSR, macrophage scavenge receptor; MSX, homeobox transcription factor muscle segment homeobox; NF, nuclear factor kappa B; OPN, osteopontin; Pit, phosphate transporter; ROS, reactive oxygen species; Runx, runt-related transcription factor; SM-MHC, smooth muscle myosin heavy chain; SOX, sex-determining region Y-box; TLR, toll-like receptor; and TNF, tumor necrosis factor.

and TNF (tumor necrosis factor)-α,[50,54,55,65,66] potentially via the Ca^{2+}-reactive oxygen species-NFκB-axis or inflammasome activation.[56,67–69] Knockdown of the toll-like receptor 4 (*TLR4), RANKL*, or *CaSR* gene abrogates secretion of TNF-α and IL-1β after CPP exposure, indicating a paramount role for TLR4, RANKL, and CaSR in CPP-induced cytokine responses.[54,56,65] Primary CPPs promote the release of IL-1β, whereas secondary CPPs induce TNF-α secretion,[54] suggesting that primary and secondary CPP have distinct receptor binding affinities and evoke distinct signaling cascades. Nonetheless, inflammasome activation is required for CPP-induced cytokine expression, as blocking inflammasome assembly abrogates overall cytokine expression (Figure 1D).[70]

Endothelial Dysfunction

The endothelium represents a barrier between circulating CPPs and underlying vascular tissue and are the first cell population exposed to CPPs upon their formation. Endothelial inflammatory activation and endothelial dysfunction are triggered by proatherogenic and proinflammatory signaling molecules and key in the development of atherosclerosis and vascular calcification (reviewed in Gimbrone and García-Cardeña,[71] Davignon and Ganz,[72] Karwowski,[73] and Boström[74]). Understanding how CPPs affect EC behavior[75] may partly explain how CPPs contribute to these and possibly other vascular pathologies.

Endothelial dysfunction is defined as the pathological state wherein vasoconstriction occurs as a consequence of an imbalance in the relative contribution of endothelium-derived relaxing and contracting factors.[76] It is well established that proatherogenic signaling molecules, including oxidized lipids, evoke endothelial dysfunction,[72] which may culminate in hypertensive responses.[77,78] CPP number and serum calcification propensity both associate with blood pressure,[9,10,79,80] implying CPP may also induce endothelial dysfunction. Moreover, endothelial dysfunction associates with serum fetuin-A levels[81] and sevelamer—a calcium binder that reduces circulating CPPs[82]—preserves endothelial-dependent vasorelaxation and maintains endothelial integrity in mice with chronic kidney disease.[83] One possible mechanism by which CPP may induce endothelial dysfunction is by reducing NO bioavailability, either by repressing the expression or activity of eNOS (endothelial NO synthase),[84,85] or by the ROS-mediated scavenging of NO.[86] Alternatively, CPPs might increase levels of asymmetrical dimethylarginine, an endogenous inhibitor of NO.[87] The exact mechanism by which CPPs induce endothelial dysfunction is unknown and warrants further investigation.

Osteochondrogenic dedifferentiation

Vascular calcification is associated with the osteochondrogenic dedifferentiation of VSMCs,[88,89] induced by the proatherogenic and proinflammatory milieu.[90–92] The osteochondrogenic dedifferentiation of VSMCs is controlled by distinct transcription factors like Runx2 (runt-related transcription factor 2), Osterix, MSX2 (homeobox transcription factor muscle segment homeobox 2), and SOX9 (sex-determining region Y-box 9; reviewed in Durham et al[93]). Activation of the osteochondrogenic transcription machinery culminates in decreased expression of contractile proteins (eg, α-smooth muscle actin, smooth muscle myosin heavy chain, smoothelin, calponin) and increased expression of osteogenic markers (osteopontin, osteocalcin, alkaline phosphatase, and collagens).[94]

Another sequel of the osteochondrogenic dedifferentiation of VSMCs is excessive production of core matrisome components (ie, collagens, proteoglycans, and glycoproteins) and extracellular matrix regulators (ie, matrix metalloproteinases and metalloproteases) that contribute to blood vessel remodeling.[95,96] This further potentiates the osteochondrogenic dedifferentiation process, aggravating impairment of vascular homeostasis and resulting in a stable proatherogenic microenvironment and increased vascular stiffness.[96]

VSMC osteochondrogenic dedifferentiation may be induced by a plethora of factors, including oxidized lipids[97] and oxidative stress,[98] inflammatory cytokines,[99] growth factors,[100] hormones,[101] vitamin D,[102] and calcium phosphate crystals.[103] Hence, the use of HMG-CoA (β-hydroxy β-methylglutaryl-CoA) reductase inhibitors—more commonly known as statins—has received high interest as potential therapeutic in vascular calcification because of their lipid-lowering and anti-inflammatory effects.[104] The inhibition of cholesterol synthesis diminishes cAMP-dependent matrix calcification by VSMC[105] and mitigates inflammation-induced artery calcification in rodents[106] via mechanisms including the lowering of plasma Ca^{2+} levels,[107] the suppression of autophagy,[108] the prevention of phosphate-induced VSMC apoptosis,[109,110] and microarchitectural changes in calcium deposits.[111] Yet, clinical studies on the use of statin therapy in vascular calcification have been discordant: statins are reported to promote,[112,113] suppress,[114,115] or have no effect on vascular calcification.[116] These discrepancies may be explained by the interaction between statins and BMP (bone morphogenic protein)-2 signaling in VSMC.[117,118] The activation of BMP-2 signaling is a key event in vascular calcification as it evokes the expression of the osteochondrogenic transcription factors Runx2 and Osterix.[119,120] Indeed, the loss of the BMP-2 inhibitory molecule Smad6 culminates in the aggravation of vascular calcification.[92,121] Statins induce the expression of BMP-2[117] and BMP receptor II[118] in VSMC, which may change the calcification process. Indeed, statins promote macrocalcification of atherosclerotic plaques, irrespective of their plaque-regressing effects.[122,123] As macrocalcifications associate with plaque stability,[124] these

observations may explain why statins decrease cardiovascular risk, despite increasing vascular calcification.[124] Thus, a deeper understanding of the mechanisms underlying vascular calcification is warranted and the clinical need for new treatments remains.

It is well accepted that CPPs promote calcification by VSMCs.[2,50,62,125] However, controversy exists on the induction of osteochondrogenic dedifferentiation by CPPs. To illustrate, some studies report reduced osteochondrogenic dedifferentiation when the formation of secondary CPPs is blocked[125] or CPPs are removed from serum,[2] whereas others fail to identify osteochondrogenic gene signatures in the calcified lesions.[45]

Mechanistic insight on the interference of CPPs on the osteochondrogenic dedifferentiation of VSMC is limited, yet the elimination of CPPs from the serum of patients with end-stage renal disease (ESRD) reduces the serum capacity to induce osteochondrogenic dedifferentiation and abrogates its procalcific capacity.[2] Likewise, the addition of CPPs derived from ESRD patients to the serum of healthy blood donors promotes the osteochondrogenic dedifferentiation of VSMCs.[2] CPP-induced osteochondrogenic dedifferentiation appears restricted to secondary CPPs, as inhibiting amorphous-to-crystalline transition prevents VSMC calcification.[125] In VSMCs, CPPs provoke an increase in cell-bound calcium[50,126] and may induce osteochondrogenic differentiation via a multitude of mechanisms (Figure 1E). First, CPPs induce the expression and secretion of TNFα by VSMC,[50] which can trigger osteochondrogenic dedifferentiation via the MSX2[127] and AP-1 (activator protein 1)[128] transcriptional regulators augmenting the expression of Runx2. Second, CPPs may provoke the expression and secretion of BMP-2 by VSMC,[103] which induces osteochondrogenic dedifferentiation via increased phosphate transport,[129] resulting in endoplasmic reticulum stress and the activation of osteogenic transcription factor XBP1 (x-box binding protein 1).[130] Third, CPPs induce VSMC oxidative stress[50] which activates a multitude of downstream signaling cascades (eg, Akt [Ak-strain transforming], p38 MAPK [mitogen-activated protein kinase], and NFκB) enhancing the transcriptional activation of the osteochondrogenic differentiation program.[131–134] Alternatively, CPPs promote the secretion of IL-6 from EC,[64] which may drive the osteochondrogenic differentiation of VSMC in a STAT3 (signal transducer and activator of transcription 3)-dependent manner.[135]

Calcifying Microvesicles

Vascular calcification occurs in the extracellular space[136,137] and is initiated by the secretion of calcifying microvesicles (CMVs) from VSMC[138] and plaque macrophages,[139] which represent nucleation sites for matrix calcification.[140] Cell-derived CMVs are distinct from blood-borne CPPs. CMVs and CPPs differ in origin, size, the presence of membranous proteins and lipids, and crystallinity (Table). CMVs are a heterogeneous group of secreted vesicles, including matrix vesicles and exosomes,[157,164,165] which function to maintain mineral homeostasis. Under physiological conditions, CMVs contain inhibitors of calcification, whereas under pathogenic conditions, promoters of calcification are present.[158,159,166,167] Once released in the extracellular space, CMV aggregate by annexin-dependent tethering[158,160] and bind to matrix collagens[161] to form nucleation sites for calcification, culminating in microcalcifications,[140] which may fuse to form macrocalcifications within the vessel wall.[168]

CPPs may influence CMV-mediated calcification in several ways. First, CPPs induce apoptosis of VSMC[59] and apoptotic bodies form a nidus for calcification.[169,170] Second, CPPs cause a rise in cytoplasmic Ca^{2+},[59] and high cytosolic Ca^{2+} levels in VSMC result in the formation of procalcifying CMVs[158] (Figure 1E). Third, CPPs can be isolated from calcified atherogenic lesions[32] wherein CPPs may fuse to and integrate into the developing microcalcifications. How CPPs interfere with CMV-mediated calcification is understudied and a complete picture is lacking. Nonetheless, serum calcification propensity and CPP maturity associate with calcified lesion size,[8,171] suggesting an interaction that deserves further evaluation.

Perivascular Adipocytes and Adventitial Fibroblasts

It is increasingly recognized that the perivascular adipose tissue actively contributes to atherogenesis[172,173] and vascular calcification.[174,175] The perivascular adipose tissue, wherein perivascular adipocytes reside, is a highly metabolic tissue, which secretes a plethora of paracrine signaling molecules, including vasoactive and immunomodulatory factors.[176–178] Proatherogenic actions of perivascular adipocytes include the secretion of proinflammatory cytokines,[179] the recruitment of inflammatory cells into the vessel wall,[180] the induction of smooth muscle cell proliferation in the neointima,[181] and the activation of adventitial fibroblasts,[182] all facilitating atherogenesis. Moreover, inflammatory activation of the perivascular adipose tissue is associated with decreased plaque stability, vascular calcification, and an increased cardiovascular risk score.[174]

Adventitial fibroblasts also contribute to atherogenesis[183] and vascular calcification.[184] Stimulated by atherogenic and proinflammatory signaling molecules, adventitial fibroblasts acquire a motile myofibroblastic phenotype[185,186] and migrate into the forming neointima.[187,188] Myofibroblast are professional extracellular matrix producing cells, that facilitate neointimal growth by the secretion of collagens and other matrix components.[189] Moreover, myofibroblasts secrete a variety of

Table. Characteristics of the Various Procalcifying Particles: CaP, CPPs, and CMVs

Particle	Aliases	Origin		Size. nm	Organic profile		Mineral profile		Biologic effect	References
		Serum, Tissue, Protein	N/S		Protein	Lipid	Crystallinity (A/C)	Mineral profile		
CaP	Calcium pyrophosphate dihydrate microcrystals	?	S	1–30	—	—	C	HAP	CPP induce inflammatory signaling in macrophages	70
	Hydroxyapatite crystals, hydroxyapatite particles	?	S	15–200	—	—	C	HAP	CaP crystals induce EC toxicity and activation, osteochondrogenic dedifferentiation, and calcification	52,58
	Nanoparticulate apatite, nanosized hydroxyapatite, calcium phosphate nanoparticles	?	S	100–300	—	—	C	cHAP, HAP	CaP crystals induce VSMC toxicity	59–61
CPPs	CPPs	Serum	N/S	30–250	FetA, Alb, ApoA, GRP, MGP	—	A: CPP-I C: CPP-II	cHAP, HAP, Monetite	CPP induce inflammatory signaling, osteochondrogenic dedifferentiation and calcification	1,2,41,49,50, 55,56,125,126
	Calcium phosphate bions	Serum	N/S	100–500	FetA, Alb, ApoA	—	A: CPP-I C: CPP-II	cHAP, HAP, Calcite	Calcium phosphate bions induce EC toxicity and intimal hyperplasia	32,64
	Calcium phosphate (nano)particles	FetA	S	30–200	FetA	?	A: CPP-I C: CPP-II	HAP	Calcium phosphate (nano) particles induce VSMC toxicity, but to a lesser extent than CaP	31,63
	Calcium phosphate precipitates	Serum	N	?	FetA	?	?	?	Calcium phosphate precipitates levels associate with kidney function and vascular calcification	141
	Calcifying nanoparticles, calcified nanoparticles	Serum and tissue	S	20–1000	?	?	?	?	Calcifying nanoparticles induce vascular occlusion and calcification	62,142,143
	Fetuin-mineral complexes	FetA	S	?	FetA, Alb, MGP	?	?	?	Fetuin-mineral complex levels associate with osteoclast activity, bone resorption and vascular calcification	144–149
	Mineralo-organic nanoparticles, mineralo-protein nanoparticles	Serum	S	50–350	FetA, Alb, ApoA	—	A: CPP-I C: CPP-II	HAP	Mineralo-organic nanoparticles induce inflammatory signaling	40,66, 150–154
	Nanobacteria	Serum	S	200–500	FetA	?	C	HAP	Nanobacteria are CPP and induce calcification	51,155
	Protein-mineral complexes, protein-mineral particles	FetA	S	50–250	FetA, Alb, MGP	?	A: CPP-I C: CPP-II	?	Protein-mineral complexes are endocytosed via SRA and induce inflammatory signaling	53,54,156
CMV	Calcifying extracellular vesicles, exosomes, Matrix vesicles	Cells (VSMC, Mph)	N	30–300	Annexins, CD9, CD63	Membranous	A	$Ca_3(PO_4)_2$	CMV contain membranous lipids and amorphous calcium phosphate and localize at sited of extracellular calcification	138–140, 157–163

CPP: CPP-1: primary CPP; CPP-II: secondary CPP. ? indicates undetermined; —, negative; A, amorphous; Alb, albumin; C, crystalline; CaP, calcium phosphate crystal; cHAP, carbonate-hydroxyapatite ($Ca_{10}(PO_4)_3(CO_3)_3(OH)_2$); CMV, calcifying microvesicle; CPP, calciprotein particle; EC, endothelial cell; FetA, Fetuin-A; GRP, GLA-rich protein; HAP, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$); MGP, matrix γ-carboxylated glutamate protein; Mph, macrophage; N, natural origin; S, synthetic origin; SRA, scavenger receptor A; and VSMC, vascular smooth muscle cell.

proinflammatory cytokines,[190] which enhance endothelial dysfunction, inflammatory cell recruitment into the neointima,[191–193] and smooth muscle cell proliferation.[186] Notably, vascular calcification may not only occur in the intima or media but also occurs in the adventitia,[194] where—under conditions of hypercalcemia and hyperphosphatemia—adventitial myofibroblasts actively contribute to calcium deposition.[19]

Thus, perivascular adipocytes and adventitial fibroblasts actively contribute to atherogenesis and calcification. Hitherto, it is obscure if, and how CPPs might alter the behavior of these cells, and thus if CPPs mediate vascular pathogenesis via the perivascular adipose tissue or adventitia is unknown.

Dynamics of CPPs In Vivo

Serum CPPs can be isolated from a variety of (pre)clinical animal models[32] and patient samples by (ultra)centrifugation,[49,141,144,145,150,195] allowing analysis of their quantity, morphology, constituents, and subsequent study of their pathogenicity in in vitro or in vivo models. Alternatively, CPP formation can be replicated in vitro by the supersaturation of serum-supplemented culture medium with calcium salts and phosphates.[32,151] Primary and secondary CPPs are, respectively, synthesized by moderate and severe calcium/phosphate supersaturation of the culture medium[66,152] or short- and long-term incubation.[54] Notably, plaque-derived and synthesized CPPs show morphological and chemical resemblance.[32]

Intravenous administration of CPPs into normolipidemic rats leads to aortic neointimal lesions in 30% to 40% of rats.[64] Such preatherosclerotic niches are characterized by endothelial activation and the osteochondrogenic dedifferentiation of VSMCs, which produce abundant extracellular matrix,[64] resembling that in human atherosclerotic plaque development.[93,196] Combining CPP administration with balloon-induced vascular injury provokes development of intimal hyperplasia in 50% to 90% of animals,[32,142,143] which vary in the presence of calcium phosphate deposits,[32,64,142,143] suggesting a secondary hit (eg, dyslipidemia or a chronic low-grade inflammation) as prerequisite for vascular calcification. Intravenous CPP administration has to date only been performed in normolipidemic animals, and it remains unclear whether CPPs are involved in the transition of developing plaques to calcified plaques. Administration of CPPs into atherosclerosis-prone apoE-deficient or low-density lipoprotein receptor–deficient mice with preestablished plaques could clearly answer this question and provide new insights into how CPPs affect atherosclerotic plaque calcification.

Despite the differences between the actions of primary and secondary CPPs in vitro, administration of either CPP type culminates in a similar outcome in vivo; that is, the prevalence of intimal hyperplasia and features of neointima formation by these 2 particle types is similar.[32,64] It is tempting to speculate that the administered primary CPPs would mature into secondary CPPs in vivo, but evidence for this is lacking. Alternatively, the shape factor of toxicity of secondary CPPs may become negligible in vivo because of the adsorption of numerous serum proteins that smooth out the otherwise sharp particles.[54] In keeping with this hypothesis, mass spectrometry analysis documented a similar protein composition for primary and secondary CPPs derived from various biofluids like serum and ascites, suggestive of an identical adsorption pattern.[150]

The ability to fluorescently label CPPs by tagging fetuin-A or albumin with fluorescent dyes or generating a fluorescent-fusion fetuin-A/albumin and subsequently incorporating it into synthesized CPPs allows for their pharmacokinetic and pharmacodynamic evaluation (eg, serum half-life, biodistribution, and clearance characteristics) as well as their cellular localization at sites of vascular injury. Alternatively, fluorescent bisphosphonate labeling of calcium phosphate offers a similar strategy to track CPPs in vivo. To illustrate, the intravenous administration of fluorescently labeled CPPs in healthy normolipidemic mice suggests that CPPs have a relatively short serum half-life and are rapidly cleared by the liver and spleen.[53,54] In mice deficient in the macrophage scavenger receptor class A/macrophage receptor with a collagenous structure, administered CPPs did not accumulate in liver Kupffer cells or spleen macrophages, suggesting that clearance of CPPs is largely dependent on macrophage uptake.[53] Furthermore, in a mouse model of calcified atherosclerosis, fluorescently labeled CPPs accumulate in the vessel lumen and plaque area and colocalize to the endothelium and macrophages.[53] No CPPs were found in the arterial wall, suggesting that CPPs did not associate with VSMCs. Noteworthy, however, is that the fluorescence intensity of CPPs critically depends on the maturity of the particles and the extent of crystallinity[54] and may not provide a sufficiently strong signal for complete in vivo imaging.

Although investigations on the in vivo effects of CPPs on the vasculature are in their infancy, development of in vivo imaging tools to assess the dynamics of CPPs, their distribution, and detection of the cell types they associate with, will undoubtedly increase insight into the pathophysiological role of CPPs in the cardiovascular system. Advances in CPP imaging enable investigation of key questions about the identity of cell types affected by CPPs in vivo or whether the detrimental effects of CPPs are limited to the cardiovascular system. These developments could culminate in the development of specific therapies targeting CPPs.

Clinical Relevance of CPPs: a Biomarker and Modifiable Risk Factor for Cardiovascular Pathology

The serum of patients with ESRD, coronary artery disease, or arterial hypertension has a greater propensity to CPP formation than serum from healthy blood donors.[79] Increased propensity to generate CPPs is associated with adverse cardiovascular outcomes (ie, all-cause and cardiovascular death, myocardial infarction, and peripheral artery disease) in patients with predialysis chronic kidney

disease (CKD)[9] and ESRD, including kidney transplant recipients.[12,15] Moreover, the augmented propensity to form CPPs associates with the occurrence and progression of severe coronary artery calcifications and atherosclerotic cardiovascular events in patients with CKD stages 2 to 4.[14,171] These observations were partially verified by findings of a recent study that patients with acute coronary syndrome have higher CPP serum levels than patients with stable angina (without predialysis CKD or ESRD) and serum CPP levels correlate with the total and lipid plaque volumes.[7] Hence, serum CPP levels may be considered a surrogate marker of coronary atherosclerosis and coronary artery calcification. Meta-analyses demonstrating a link between reduced serum fetuin-A and albumin and a higher risk of coronary artery disease, additionally testify to the potential importance of elevated calcification propensity in the pathogenesis of atherosclerosis.[197,198]

A method to determine calcification propensity has been developed which may be used for diagnostic approaches; CPP formation in patient serum is induced by supersaturating the serum with calcium and phosphate and measuring the optical density after incubation (Figure 2A). Other methods to quantify CPPs in serum and biofluids include microplate-based dynamic light-scattering and electron or atomic force microscopy. Microplate-based dynamic light scattering is both a high-throughput and precise method for estimating the hydrodynamic radius of nanoparticles and can be modified to detect CPPs.[8] Alternatively, electron or atomic force microscopy are low-throughput but demonstrative methods for CPP visualization[2,49] (Figure 2B). Alternatively, one-half maximal transition time has been established as a measure of primary-to-secondary CPP transition, and a prognostic biomarker in various patient cohorts (Figure 2C).[9–15,79] Although this method provides a surrogate marker suggesting elevated CPP formation in disease, it remains unclear if all types of CPPs are equally detected, what their composition is, and whether the actual concentration of circulating CPPs is indeed elevated. Nonetheless, validation by independent groups of the association between a decreased one-half maximal transition time and the occurrence of pathology are appearing in literature.[199,200]

A recently introduced flow cytometry-based technique allows for direct quantification of CPPs in serum and other biofluids (Figure 2D), which may be translated into routine clinical diagnostics. In this protocol, CPP and membranous extracellular vesicles are separated from other cellular particulates by size-exclusion or ultracentrifugation and further characterized by a combination of a fluorescently labeled bisphosphonate (OsteoSense 680EX) that labels mineral deposits and a green fluorescent membrane-intercalating dye (PKH67) that labels membranous structures. Using this technique, CPPs are detected as OsteoSense$^+$/PKH67$^-$ events, whereas calcifying extracellular vesicles appear as OsteoSense$^+$/PKH67$^+$ events.[7,162] Moreover, CPPs can be further discriminated on basis of their light-scattering properties, allowing for the separate quantification of primary and secondary CPPs[162] (Figure 2D).

The clinical significance of serum CPPs is highlighted by the recent TACT (Trial to Assess Chelation Therapy; https://www.clinicaltrials.gov; Unique identifier: NCT00044213). Serum CPPs can be routinely decalcified using EDTA disodium salt in vitro, and infusion of EDTA culminates in reduced cardiovascular risk in patients. In TACT, the EDTA treatment regimen was associated with 1.22-fold lower risk of a primary composite end point (death from any cause, repeated myocardial infarction, stroke, coronary revascularization, or hospitalization for angina pectoris).[201] Notably, in subgroups of patients with diabetes,[202] and those having diabetes mellitus and peripheral artery disease—2 conditions whereby patients have elevated serum CPP levels—the reduction in risk scores was even greater (1.69- and 1.92-fold, respectively).[203] Although EDTA therapy is relatively safe,[204] its limited bioavailability (≈5%) when taken orally[205] limits its clinical use. Follow-up trials (TACT2 [Trial to Assess Chelation Therapy-2; https://www.clinicaltrials.gov; Unique identifier: NCT02733185] and TACT3a [Trial to Assess Chelation Therapy-3a; https://www.clinicaltrials.gov; Unique identifier: NCT03982693] trials) are ongoing, focused on the efficacy of chelation therapy specifically in diabetic patients with prior myocardial infarctions and individuals with diabetes and critical limb ischemia resulting from severe peripheral atherosclerosis, respectively. Besides chelation therapy, new clinical studies are starting that specifically aim to reduce the serum calcification propensity or the number of circulating CPPs.[82,206,207] Albeit their initial data indicates a successful reduction in CPP formation, their effects on long-term cardiovascular risk have yet to become apparent.

Future Perspectives and Therapeutic Implications for CPPs in Cardiovascular Pathology

The clinical relevance of elevated circulating CPP levels is illustrated by a significant correlation between an augmented calcification propensity or increased number of circulating CPPs and a higher risk of adverse outcomes, including major cardiovascular events and mortality.[9–14] As CPPs represent a modifiable risk factor for cardiovascular diseases, pioneering clinical trials aimed at reducing the level of circulating CPPs are ongoing.[82,206,207] Despite current advances in CPP research, revealing their clinical relevance to cardiovascular morbidity and primary modes of action, many questions remain unanswered.

First, we propose that the methods for obtaining CPPs require standardization, as their current nomenclature (Table), isolation techniques, and synthesis methods are diverse. CPP extraction from biological fluids is currently

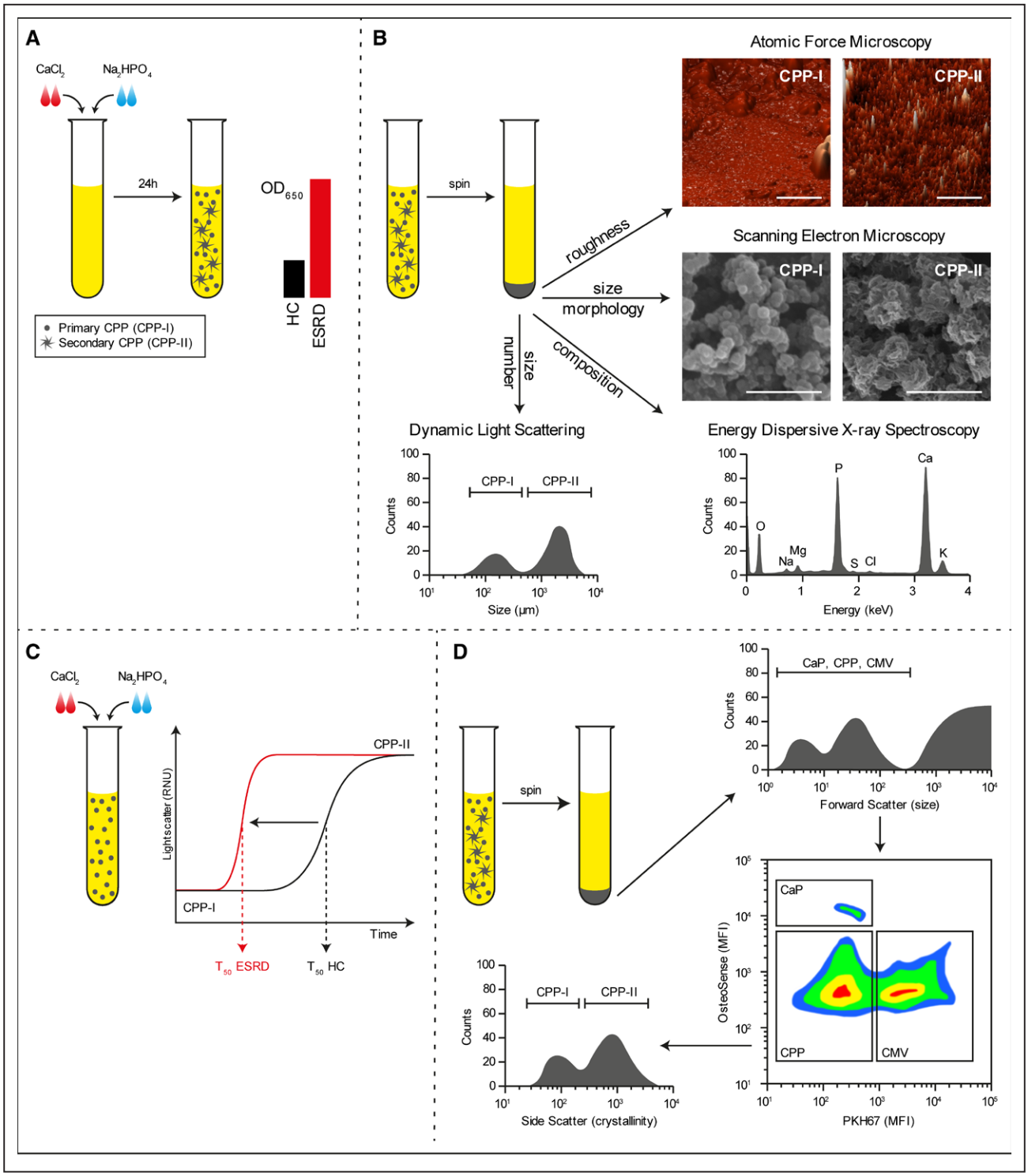

Figure 2. Methods to detect calciprotein particles (CPPs) in clinical samples.
Supersaturation of serum with calcium chloride ($CaCl_2$) and sodium diphosphate (Na_2HPO_4) followed by incubation under culture conditions for 24 h causes the formation of CPPs that can be measured by absorbance at 650 nm. In disease conditions wherein CPP levels are increased, the OD_{650} readings increase (**A**). Alternatively, CPPs can be pelleted by centrifugation and investigated by dynamic light scattering to assess particle size, electron and atomic force microscopy to assess morphology, or elemental analysis (EDX) to assess mineral constituent (**B**). Supersaturation of serum is also used to measure the one-half maximal transition time needed for amorphous-to-crystalline transition (T_{50}). An increased serum propensity for secondary CPP formation is observed as a reduction in T_{50} (**C**). A novel flow cytometry-based technique allows for the direct quantification of CPP levels in serum. Here, serum precipitates are labeled with a combination of a fluorescent bisphosphonate (osteoSense) and a fluorescent membrane-intercalating dye (PKH67) and separated based on size, calcium phosphate content, and the presence of membranous lipids. CPPs are observed as OsteoSense$^+$/PKH67$^-$ events that fluoresce dim compared to calcium phosphate crystal (CaP) crystals. CPPs are further characterized as primary- or secondary CPPs based on crystallinity (**D**). CMVs indicates calcifying microvesicles; ESRD, end-stage renal disease sample; HC, healthy control sample; MFI, mean fluorescence intensity; and OD, optical density.

limited to the serum of etidronate-, vitamin D–treated, or uremic rats,[141,144–148,156] with only few studies reporting the isolation of CPPs from human blood or tissue.[2,32] Moreover, the pathogenic capacity of CPPs may depend on the health of the serum donor. Although CPPs can be synthesized in vitro by combining serum, Ca^{2+}, and PO_4^{3-}, a systematic and detailed comparison of CPPs synthesized using serum from cardiovascular patients and CPPs produced using serum from healthy human volunteers is lacking. We recommend performing in-depth characterization of CPPs' physicochemical properties (eg, Ca^{2+}, phosphate and protein content, particle size, and crystallinity) and comparing them to native CPPs isolated from patient sera, before using in vitro synthesized CPPs for mechanistic studies. Moreover, rather than the current multitude of protocols used to synthesize CPP in vitro, the research field would benefit from standardization.

Second, the current classification of CPPs into either primary (amorphous) or secondary (crystalline) particles may be oversimplified. CPPs can adsorb macromolecules from the ambient fluid and undergo dissolution-reprecipitation and ion exchange reactions.[150,153,154,208] This leads to formation of a variety of different particles, not limited to certain sets hitherto defined as primary or secondary CPPs. Moreover, the exact shape, crystallinity, and chemical composition of CPPs within tissues are affected by several local factors including pH, amount, and relative proportion of available mineral ions,[209] and the conformation of CPPs present in the vascular tissues they affect remains unclear. We strongly recommend comprehensive mineral and organic profiling as CPP effects, and their molecular mechanisms are defined by these physical and chemical features. This profiling would preferentially include the visualization of CPP size, structure, shape, crystallinity, and chemical composition combined with mass spectrometry approaches to determine the protein composition.

Third, it remains unclear whether particle formation under conditions of hyperphosphatemia is restricted to Ca^{2+} and whether alternative protein-mineral particles have pathophysiological properties like those of CPPs. Comparing the pathogenic effects of magnesium phosphate particles with the same size, shape, and organic profile as CPPs, we found that, unlike CPPs, these particles lack pathogenic capacity, suggesting that the pathogenic potential of CPPs is defined by its mineral component and possibly its crystallinity and not its proteinaceous constituents.[64] Moreover, administration of CPPs produced using pyrophosphate—a phosphate substitute that does not allow for hydroxyapatite crystal formation—causes no pathogenic effects, suggesting that the specific crystals, and not the Ca^{2+} or phosphate, possess pathogenic capacity.[210]

Fourth, current understanding of the signaling mechanisms evoked by CPP exposure is inadequate. Valuable information on the signaling mechanisms underlying CPP-mediated pathogenesis has been obtained from in vitro experiments (discussed in this review), but the observation that CPPs induce massive cell death in vitro but not in vivo suggests that CPP may evoke different signaling events in vitro and in vivo and may explain why current methodologies have been unable to identify clear alterations in signaling pathways. This illustrates the need to develop in vitro systems that mimic pathophysiology more closely. Furthermore, recent advances in high-throughput "-omics" approaches (RNA-sequencing, ribosome profiling, and mass spectrometry) will in the future provide a better insight into CPP-mediated signaling in primary vascular cells, as the lack of such data currently inhibits our understanding of cell-specific effects of CPPs and their involvement in pathogenesis. We propose that using single-cell RNA-sequencing can separate the process of cell death and other signaling events after exposure of vascular cell populations to CPPs. This approach can be complemented by combining CPP exposure with established cardiovascular risk factors (hypoxia, oxidized low-density lipoprotein cholesterol, advanced glycation end-products).

Regarding the in vivo studies reported to date, CPPs display different pathogenic behavior in animals and humans. In humans, elevated levels of CPPs have been primarily associated with increased vascular calcification,[3,9,149] whereas in rodents CPP administration is associated with intimal hyperplasia and atherosclerosis[64] and a highly variable frequency of vessel calcification.[32,64,142,143] It should, however, be noted that the animal models currently used for CPP administration are normolipidemic, without a renal phenotype. Performing further studies to investigate the ability of CPPs to induce or aggravate vascular calcification would best be conducted in animal models that are predisposed to vascular calcification, such as partially nephrectomized rodents, or animals with dyslipidemia or inherently disturbed mineral homeostasis.

From clinical perspective, the elevation of circulating CPPs levels in patients with acute coronary syndrome compared with those with stable angina suggest possible importance of this parameter to prognosticate ischemic heart disease. Circulating CPP levels may also have prognostic value in other patient cohorts, including individuals with osteopenia/osteoporosis, primary hyperparathyroidism, or CKD, as these conditions are characterized by hypercalcemia and hyperphosphatemia, and the concentration of CPPs in the blood is closely reflected by patients' mineralization status. As such, investigations into circulating CPP levels may explain the relationship between elevated bone turnover and the increased risk of cardiovascular disorders observed in these patients. Also, noteworthy, however, is that current investigations have focused primarily on measurement of calcification propensity rather than on direct detection of CPPs in the blood. The number of circulating CPPs may better predict cardiovascular

outcomes in these patients and would be a valuable addition to measuring calcification propensity.

From a translational perspective, pioneering studies using chelation therapy have established that circulating CPPs indeed represent a modifiable risk factor for cardiovascular outcome, although generalized chelation therapy has its limitations. Future research should focus on identifying Ca^{2+} chelators with a superior pharmacokinetic profile, or medicaments to facilitate the hepatic clearance of CPPs in patients at risk of developing cardiovascular events. For instance, Mg^{2+} has been recently suggested as a promising new therapeutic intervention in the development of CPP-induced vascular calcifications, as it dose-dependently delays maturation from primary to secondary CPPs and prevents VSMC calcification in vitro.[125] Mg^{2+}-supplementation prevents and reverses the development of vascular calcifications in mice,[211] making it a promising therapeutic intervention for patients with increased CPP levels.[212] Replacement of calcium carbonate with lanthanum carbonate lowers serum CPP levels in patients with ESRD,[213] which may explain its beneficial effect on the attenuation of aortic calcification.[214] A recent study proposed 4,6-di-O-(methoxy-diethyleneglycol)-myo-inositol-1,2,3,5-tetrakis(phosphate)—an inositol phosphate analog—as an agent limiting primary-to-secondary CPP transition and preventing vascular calcification.[215] These results suggest avenues for future clinical trials of crystallization inhibitors specifically targeting the formation of harmful secondary CPPs, at least in high-risk patients with CKD.

CONCLUSIONS

CPPs may be proposed as a relatively novel potential culprit of vascular disease which can be particularly important in patients with a concomitant chronic kidney disease. Yet, exactly how CPPs influence vascular cells and cardiovascular pathology in vitro and vivo remains obscure. Upcoming research may uncover additional detrimental effects of CPPs, or pathways mediating the underlying pathophysiological mechanisms, whereas clinical investigations aim at direct identification of CPPs in the serum to evaluate their association with various cardiovascular pathologies. New insights into CPP-induced cardiovascular pathology will certainly lead to improved therapeutic interventions and possibly benefit cardiovascular outcome.

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Affiliations

Laboratory for Vascular Biology, Division of Experimental and Clinical Cardiology, Research Institute for Complex Issues of Cardiovascular Diseases, Kemerovo, Russian Federation (A.G.K., A.E.K., A.E.Y.). Department of Pathology and Medical Biology, Division of Pathology (L.F., J.-L.H.) and Laboratory for Cardiovascular Regenerative Medicine, Department of Pathology and Medical Biology (L.F., G.K.), University Medical Center Groningen, University of Groningen, the Netherlands. Sulfateq B.V., Admiraal de Ruyterlaan 5, 9726 GN, Groningen, the Netherlands (G.K.).

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Disclosures

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