Chronic Kidney Disease Circulating Calciprotein Particles and Extracellular Vesicles Promote Vascular Calcification: A Role for GRP (Gla-Rich Protein)

Chronic Kidney Disease Circulating Calciprotein Particles and Extracellular Vesicles Promote Vascular Calcification

A Role for GRP (Gla-Rich Protein)

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Objective—Inhibition of mineral crystal formation is a crucial step in ectopic calcification. Serum calciprotein particles (CPPs) have been linked to chronic kidney disease (CKD) calcification propensity, but additional knowledge is required to understand their function, assemblage, and composition. The role of other circulating nanostructures, such as extracellular vesicles (EVs) in vascular calcification is currently unknown. Here, we investigated the association of GRP (Gla-rich protein) with circulating CPP and EVs and the role of CKD CPPs and EVs in vascular calcification.

Approach and Results—Biological CPPs and EVs were isolated from healthy and CKD patients and comparatively characterized using ultrastructural, analytic, molecular, and immuno-based techniques. Our results show that GRP is a constitutive component of circulating CPPs and EVs. CKD stage 5 serum CPPs and EVs are characterized by lower levels of fetuin-A and GRP, and CPPs CKD stage 5 have increased mineral maturation, resembling secondary CPP particles. Vascular smooth muscle cell calcification assays reveal that CPPs CKD stage 5 and EVs CKD stage 5 are taken up by vascular smooth muscle cells and induce vascular calcification by promoting cell osteochondrogenic differentiation and inflammation. These effects were rescued by incubation of CPPs CKD stage 5 with γ-carboxylated GRP. In vitro, formation and maturation of basic calcium phosphate crystals was highly reduced in the presence of γ-carboxylated GRP, fetuin-A, and MGP (matrix gla protein), and a similar antimineralization system was identified in vivo.

Conclusions—Uremic CPPs and EVs are important players in the mechanisms of widespread calcification in CKD. We propose a major role for cGRP as inhibitory factor to prevent calcification at systemic and tissue levels.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2018;38:575-587. DOI: 10.1161/ATVBAHA.117.310578.)**

Key Words: cardiovascular disease ■ extracellular vesicles ■ myocytes, smooth muscle ■ nanoparticle ■ renal insufficiency, chronic ■ vascular calcification

Chronic kidney disease (CKD) patients develop extensive and progressive vascular calcification (VC) that contributes to the high cardiovascular morbidity and mortality.[1] It is currently known that multiple overlapping systemic and local inhibitory networks, functioning in highly complex and interconnected active processes, have evolved to prevent deposition of mineral at ectopic sites.[2,3] Features such as loss of anticalcific mechanisms, vascular smooth muscle cell (VSMC) differentiation, chronic inflammation, increased extracellular matrix remodeling, and the release of extracellular vesicles (EVs) are known features involved in the development of calcific lesions.[2–6] In addition to the presence of traditional cardiovascular risk factors, disturbances in mineral metabolism such as elevated calcium (Ca), phosphorous (P) and Ca×P product, often associated with the uremic milieu, also contribute to VC and the increased cardiovascular mortality in CKD.[2,7] Moreover, even in healthy conditions, serum is near supersaturation with respect to Ca and P and inhibitory mechanisms must exist to prevent extraskeletal mineralization. The discovery of a fetuin–mineral complex also known as calciprotein particle (CPP) in circulation, predominantly composed of Ca and P mineral, fetuin-A, and calcium-regulatory proteins, highlights a possible mechanism by which ectopic mineralization is prevented.[8,9] Fetuin-A acts as a potent inhibitor of ectopic calcification through the binding of small clusters of calcium and phosphate preventing its growth, aggregation, and precipitation.[9,10] Fetuin-A and other proteins contained in this soluble protein mineral particle (CPP) can be considered as mineral chaperons with a role in stabilization, transport, and recycling of water-insoluble mineral in

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Nonstandard Abbreviations and Acronyms

αSMA	α-smooth muscle actin
BCP	basic calcium phosphate
Cox2	cyclooxygenase-2
cGRP	γ-carboxylated GRP
CKD	chronic kidney disease
CKD5	chronic kidney disease stage 5
CPP	calciprotein particle
CPP5	calciprotein particles isolated from CKD5 patients
CPPI	primary calciprotein particles
CPPII	secondary calciprotein particles
EVs	extracellular vesicles
EVs5	extracellular vesicles isolated from CKD5 patients
GRP	gla-rich protein
IL-1β	interleukin 1β
MGP	matrix gla protein
OPN	osteopontin
Runx2	runt-related transcription factor 2
TEM	transmission electron microscopy
ucGRP	undercarboxylated GRP
VC	vascular calcification
VSMCs	vascular smooth muscle cells
WB	Western blot

blood.[9,10] The inhibition of mineral crystal formation is a crucial step to prevent the deleterious effects of calcium crystals and ectopic calcification, either in circulating CPPs or in EVs. Correlations between CPP levels and CKD development, outcome, and VC have been reported, and several in vitro experiments have shown a deleterious effect of synthetic CPPs in cultured cells, promoting inflammatory and mineralization responses.[11–16] Nevertheless, it is currently unknown whether these in vivo particles are themselves pathogenic and directly involved in VC. Moreover, additional characterization on the composition and mechanisms involved in natural occurring CPPs formation is required.

It is widely accepted that most cells release EVs that can eventually reach circulation.[17] In addition to the well-known role in the initiation of either physiological or pathological calcification,[18–21] EVs have been considered master players in intercellular communication with the capacity to transfer their internal cargo to recipient cells affecting and altering their normal functioning. EVs are currently considered a rich source of biomarkers in several pathological conditions and promising drug delivery systems with high therapeutic potentialities.[17,21–23] Although the role of VSMCs-released EVs in VC has been highlighted in recent years,[4,18–20] the potential relationship between EVs present in circulation and ectopic calcification at tissue level is mostly unknown, as well as their potential use as biomarkers for VC.

We have shown that the vitamin K–dependent GRP (Gla-rich protein) functions as an inhibitor of calcification in the cardiovascular[24] and articular systems.[25] GRP calcium-binding properties[26,27] and its association to calcification processes[24–29] indicate that its function might be associated with prevention of calcium-induced signaling pathways and direct mineral binding to inhibit crystal formation/maturation. GRP is also involved in the mineralization competence of VSMC-derived EVs and possibly associated with the fetuin-A/MGP (matrix gla protein) calcification inhibitory system.[24] In addition, GRP was shown to function as an anti-inflammatory agent in articular and immune cells able to decrease proinflammatory responses, suggesting a role as crosstalk agent between calcification and inflammatory processes.[25,30] In this work, we investigated the role of GRP in CPP formation and its association with circulating EVs and CPPs in CKD. Also, we studied the pathogenic effect of CKD-derived CPPs and circulating EVs in VSMCs calcification. Additionally, the relevance and function of GRP for mineral crystal formation and maturation was highlighted.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

CPP-Like Entities Are Present in Serum of Healthy Control Individuals

CPP particles have been isolated from the serum of patients with pathological situations involving imbalanced mineral metabolism, such as CKD, but not from healthy individuals.[9,11,12,31] However, SDS-PAGE of isolated CPPs from controls and CKD patients revealed a high protein content in control CPPs, with significant differences in total protein profile when compared with those isolated from all CKD stages analyzed (Figure 1A). Under native PAGE conditions, CPPs from controls behave as a single band (Figure 1B), which is indicative of a major protein complex. Because, by definition, CPPs are composed of a calcium phosphate mineral complex and calcium-regulatory proteins such as fetuin-A, we investigated the existence of a mineral phase and the presence of fetuin-A and GRP in control CPPs. For that, mineral-enriched and protein-enriched fractions were obtained from a pool of control CPPs by extraction with 6 mol/L GuHCl using a procedure similar to that used to extract the organic matrix of calcified tissues.[24,26] The resulting mineral-containing pellet was analyzed by micro Fourier transform infrared spectroscopy and showed an intense PO_4^{3-} absorption band in the region 1000 to 1100 cm^{-1}, characteristic of hydroxyapatite (Figure 1C).[32] Inductively coupled plasma analysis confirmed the presence of Ca in this mineral-containing fraction (results not shown). The SDS-PAGE protein profile of extracted CPPs proteins and the sample without protein extraction (CPP) suggest that the proteins associated with the mineral complex were successfully extracted (Figure 1D). Western blot (WB) analysis confirmed the presence of fetuin-A and GRP (Figure 1E). The protein composition of control CPP was characterized by liquid chromatography-tandem mass spectrometry after both chymotrypsin and a combination of endoproteinase-Lys-C-trypsin digestion. Combined analysis of both samples resulted in the identification of 649 proteins (Table I in the online-only Data Supplement), including fetuin-A, fibronectin 1, albumin, fibrinogen, β-actin, apolipoprotein A1, SPP24 (secreted phosphoprotein 24), PF4 (platelet factor 4), and prothrombin, all previously described to be

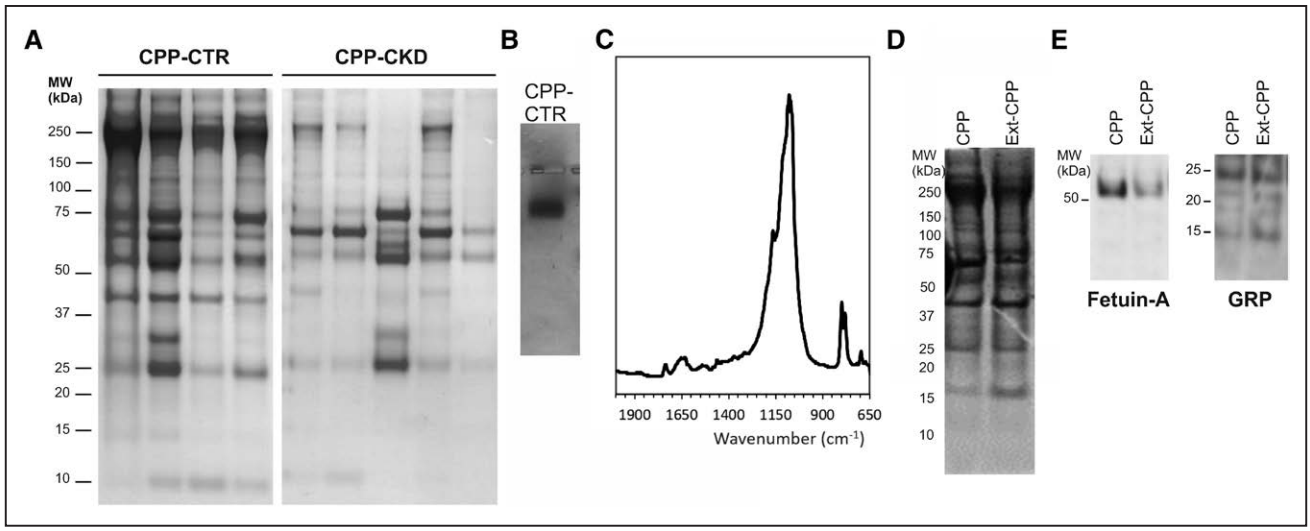

Figure 1. Calciprotein particles (CPPs) containing a mineral phase and a protein complex with fetuin-A and GRP (Gla-rich protein) are present in control (CTR) individuals. **A**, Representative SDS-PAGE gel followed by coomassie brilliant blue (CBB) staining of several control and chronic kidney disease (CKD)–derived CPPs showing the global protein profile. Molecular weight markers are indicated on the left side (kDa). **B**, CTR-derived serum CPP sample analyzed by native gel electrophoresis and stained with CBB. **C**, Fourier transform infrared spectroscopy analysis of the mineral phase of CPPs, isolated from a pool of CTR serum, after protein extraction with 6 mol/L guanidinium hydrochloride. **D**, Protein profile analyzed by SDS-PAGE and CBB staining of CPPs after centrifugation at 17 000*g* (CPP), and proteins extracted with 6 mol/L guanidinium hydrochloride (Ext-CPP). **E**, Western blot analysis of fetuin-A and GRP from protein extract gel shown in (**D**), using the fetuin-A (Santa Cruz Biotechnology) and CTerm-GRP antibodies, respectively.

associated with CPPs.[11,33] The identified proteins were found to be associated with diverse biological functions (Figure I in the online-only Data Supplement), and several calcium-binding, calcification inhibitors and proteins involved in bone mineralization were identified. In particular, several annexin proteins A1, A3, A5, A6, and A11, described to participate in the formation of hydroxyapatite crystals and with known functions in the VSMC EV-mediated mineralization process,[4,5,34] were present in control CPPs.

CPPs From CKD Are Characterized by Reduced Levels of GRP and Fetuin-A and Increased Mineral Crystal Maturation

To study a possible relationship between CPP particles and CKD, a biochemical comparative analysis between controls and CKD CPPs was performed. As already suggested by the SDS-PAGE of the protein profile (Figure 1A), total protein quantity present in the CPP complexes is significantly decreased in CKD stage 5 (CKD5) patients relatively to controls (Figure 2A). No differences were obtained in the amount of calcium associated with CPP between controls, CKD stage 4, and CKD5 (Figure 2B). WB analysis of CPP radioimmunoprecipitation assay extracts show decreased levels of fetuin-A and GRP in CPPs from CKD5 patients relative to the control group (Figure 2C). A specific sandwich ELISA for GRP was developed and validated (Figure II in the online-only Data Supplement). Levels of GRP and fetuin-A were lower in the radioimmunoprecipitation assay extracts of CPP from CKD5 patients compared with controls (Figure 2D).

Because calcium levels were similar between controls and CKD-derived CPPs, but levels of GRP and fetuin-A associated with CKD CPPs were reduced, thereby suggesting a decreased calcification inhibitory capacity in CKD CPPs, we investigated morphological differences between CPPs from controls and CKD5 patients. Transmission electron microscopy (TEM) analysis revealed that control-derived CPPs display clusters of spherical aggregates with distinguishable spheroid particles, characteristic of primary CPP (CPPI) particles. However, CPPs from CKD5 patients showed an increased amount of larger needle-like structures, morphologically compatible with secondary calciprotein (CPPII) calcium phosphate crystals (Figure 2E). Spherical particles with a lipid bilayer were only sporadically visualized indicative of low microvesicle content (results not shown). Both control and CKD5 CPPs were shown to contain Ca/P as detected by energy-dispersive x-ray spectroscopy (Figure III in the online-only Data Supplement).

Control and CKD-Derived EVs Are Differently Loaded With Calcification-Related Markers

Because EVs can be released into the blood stream and calcification-related markers such as GRP and fetuin-A are present in VSMC-derived EVs,[4,5,24] we investigated a possible relation between circulating EVs and CKD pathology.

To determine the best methodology to isolate serum-derived EVs, different approaches were initially tested by using polymer-based reagents and classical ultracentrifugation. TEM analysis showed highly heterogeneous samples from polymer isolated EVs, with high levels of protein contaminants (results not shown), and the exosomal marker CD9 was detected on the expected molecular weight only in the ultracentrifugation-processed samples (Figure IV in the online-only Data Supplement). Therefore, ultracentrifugation was the methodology used for all further experiments involving EVs characterization.

EVs isolated from control and CKD individuals were morphological and biochemically characterized (Figure V in

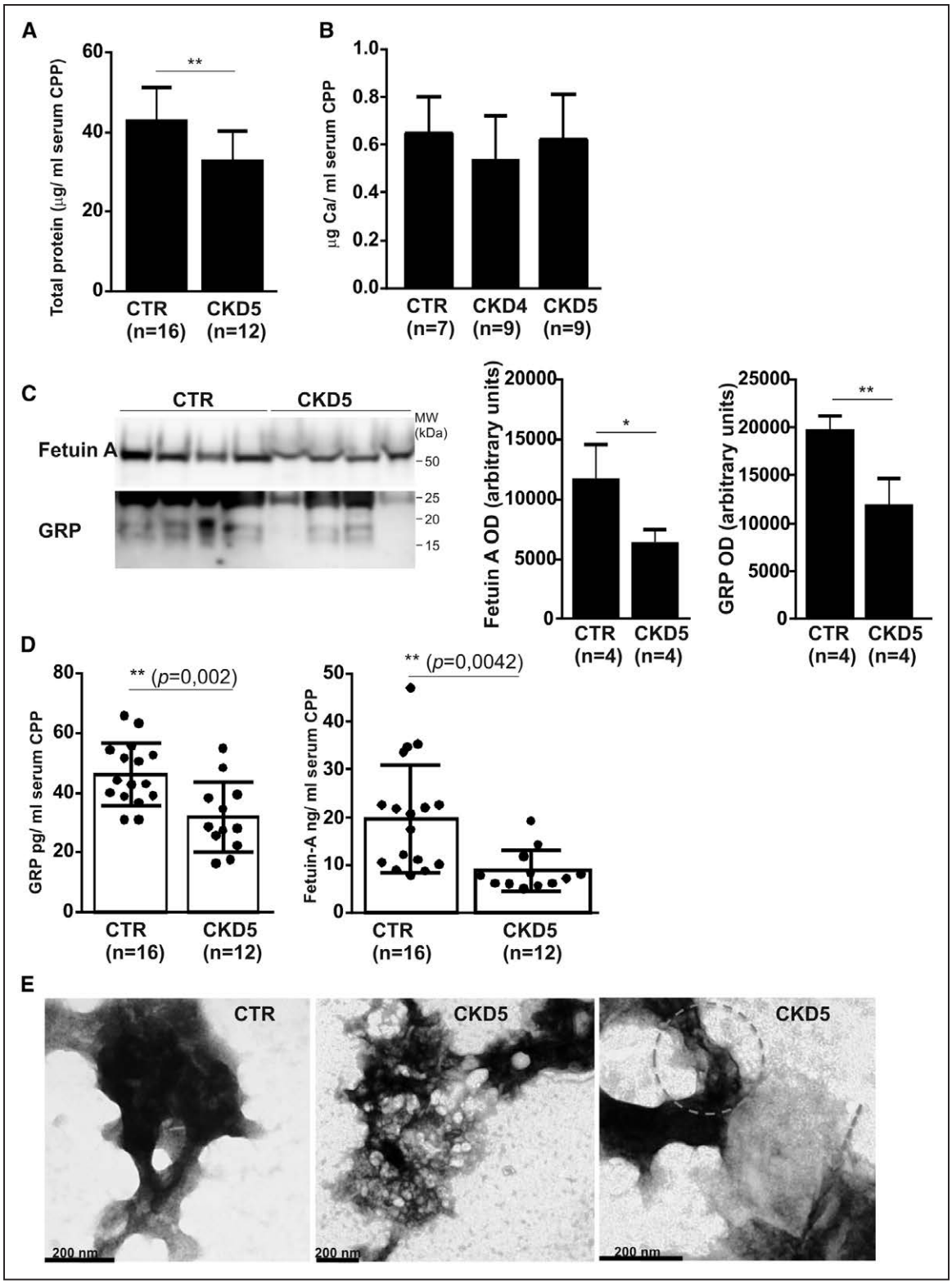

Figure 2. Comparative analysis of control and chronic kidney disease (CKD) serum-derived calciprotein particles (CPPs). **A**, Total protein quantification in CPP radioimmunoprecipitation assay extracts obtained from CTR and CKD5 serum. **B**, Calcium quantification by inductively coupled plasma from CPPs isolated from control (CTR), CKD stage 4 (CKD4), and CKD5 patient's serum. **C**, Western blot analysis of CTR and CKD5 CPPs using the CTerm-GRP (Gla-rich protein) polyclonal antibody (GenoGla Diagnostics) and the fetuin-A monoclonal antibody (Santa Cruz Biotechnology). Molecular weight markers are indicated on the right side (kDa). Quantification of protein levels was performed by measuring optical densities (OD) using ImageJ software and are presented as arbitrary units. Unpaired *t* tests were used. Statistical significance was defined as $P \leq 0.05$ (*) and $P \leq 0.01$ (**). **D**, Quantification of GRP and fetuin-A using specific sandwich ELISA assays from CPP samples described in (**A**). Unpaired *t* tests were used. Statistical significance was defined as $P \leq 0.01$ (**). **E**, Representative negative staining transmission electron microscopy images of CTR and CKD5 CPPs. Circle and arrow denote regions with clearly distinguishable needle-like structures.

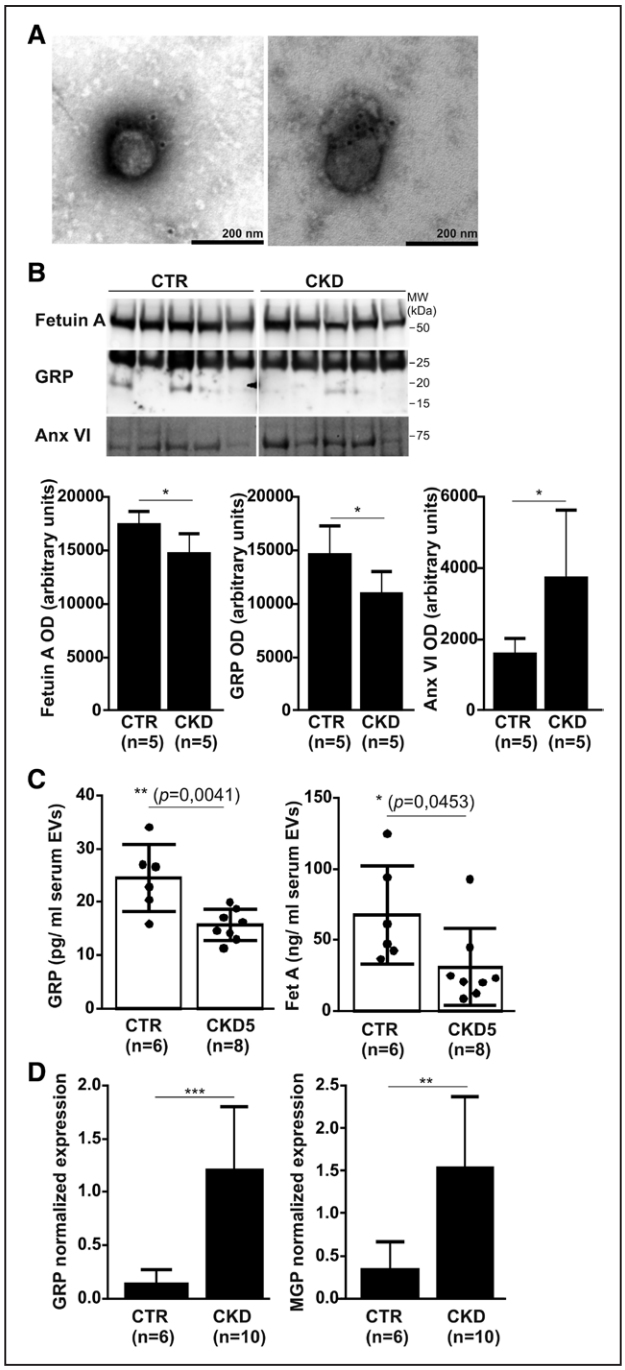

Figure 3. Control and chronic kidney disease (CKD) circulating extracellular vesicles (EVs) are differently loaded with calcification-related markers. **A**, Representative transmission electron microscopy images of immunogold staining of GRP in isolated EVs using the CTerm-GRP (Gla-rich protein) antibody. **B**, Twenty-five micrograms of EVs radioimmunoprecipitation assay protein extracts isolated from control and several CKD stage samples were analyzed by Western blotting for detection of GRP and fetuin-A as described in the legend of Figure 2 and for detection of annexin 6 (Anx VI) using a monoclonal antibody (Santa Cruz Biotechnology). Quantification of protein levels was performed as described in Figure 2 legend. **C**, Quantification of GRP and fetuin-A using specific sandwich ELISA as described in the legend of Figure 2 from EV isolated from CTR and CKD stage 5 (CKD5) serum samples. Unpaired *t* tests were used. Statistical significance was defined as $P \leq 0.05$ (*) and $P \leq 0.01$ (**). **G**, Levels of GRP and MGP (matrix gla protein) mRNAs in EVs (*Continued*)

the online-only Data Supplement). Measurement of particle size revealed the presence of 2 distinct populations in both control and CKD5 samples. The most abundant population in both control and CKD5 EVs were similarly constituted by particles of ≈150 nm (>90%) and a small percentage of population (<7.5%) with particles of <5000 nm in size (Figure VA in the online-only Data Supplement). TEM analysis showed the presence of small intact vesicles slightly varying in size (Figure VB in the online-only Data Supplement) and sporadically some vesicles aggregation (results not shown), likely representing the population with higher average size. WB showed positive detection of the exosomal markers CD9 and TSG101 (Figure VC in the online-only Data Supplement). Although CD9 does not differ between control and CKD5, levels of TSG101 were found lower in the CKD5 group relatively to control. Protein quantification revealed similar total protein levels between control and CKD5 groups (Figure VD in the online-only Data Supplement), and nonsignificant differences were found for calcium quantification (Figure VE in the online-only Data Supplement).

TEM immunogold staining detected GRP in circulating EVs (Figure 3A). Levels of fetuin-A, GRP, and annexin A6 (Anx VI) were evaluated by WB in several control and CKD samples from different disease stages and showed lower levels of fetuin-A and GRP and increased levels of Anx VI in EVs isolated from the CKD group (Figure 3B). Quantification of GRP and fetuin-A by ELISA confirmed significant lower levels of both proteins in the CKD5 group (Figure 3C).

Because EVs, particularly exosomes, are known to carry nucleic acids including mRNA, we searched for the presence and expression patterns of GRP and MGP in control and CKD-isolated EVs. RNase A treatment of EVs confirmed the intravesicular origin of RNA (Figure VIA and VIB in the online-only Data Supplement), and GAPDH with constant C_t (threshold cycle) values was used as normalizing housekeeping gene (Figure VIC in the online-only Data Supplement). Quantification of GRP and MGP expression revealed higher levels of both these mRNAs in CKD-derived EVs (Figure 3D). Because it has been shown that many mRNA molecules in exosomes are only present as fragments and are consequently nonfunctional when transferred to target cells, we amplified and confirmed the presence of the complete GRP open-reading frame in control isolated EVs (results not shown).

Calcification Potential of Uremic Serum in VSMC Is Decreased After Removal of CPP and EV Particles

To investigate a possible relationship between serum CPP and EV particles with VC, we treated VSMCs with elevated Ca/P in the presence of different variations of human serum either containing or depleted of these nanostructures and evaluated the effect on mineralization, osteogenic differentiation, and inflammation. As expected, complete serum (whole serum)

Figure 3 Continued. isolated from control and CKD patients were determined by quantitative polymerase chain reaction, and normalized fold expression (arbitrary units) using GAPDH as normalizing gene was determined relative to zero. Data are presented as mean±SE. Unpaired *t* tests were used. Statistical significance was defined as $P \leq 0.05$ (*) and $P \leq 0.01$ (**).

from CKD5 patients increased VSMC calcification compared with whole serum from the control group (Figure 4A), characterized by increased expression of the osteogenic markers Runx2 (runt-related transcription factor 2) and OPN (osteopontin; Figure 4B and 4C). Removal of either CPP or EV particles from CKD5 serum resulted in a strong reduction of VSMC calcification (Figure 4A). Interestingly, removal of CPP from control serum does not affect calcification, whereas removal of EVs strongly increased calcification, both in VSMCs (Figure 4A) and in aortic segments ex vivo culture (Figure VII in the online-only Data Supplement), suggesting a protective effect of EVs in physiological conditions. Increased VSMC calcification on removal of control EVs is characterized by decreased levels of αSMA (α-smooth muscle actin; Figure 4D) and increased levels of the osteogenic marker Runx2 (Figure 4B) and inflammation marker genes IL-1β

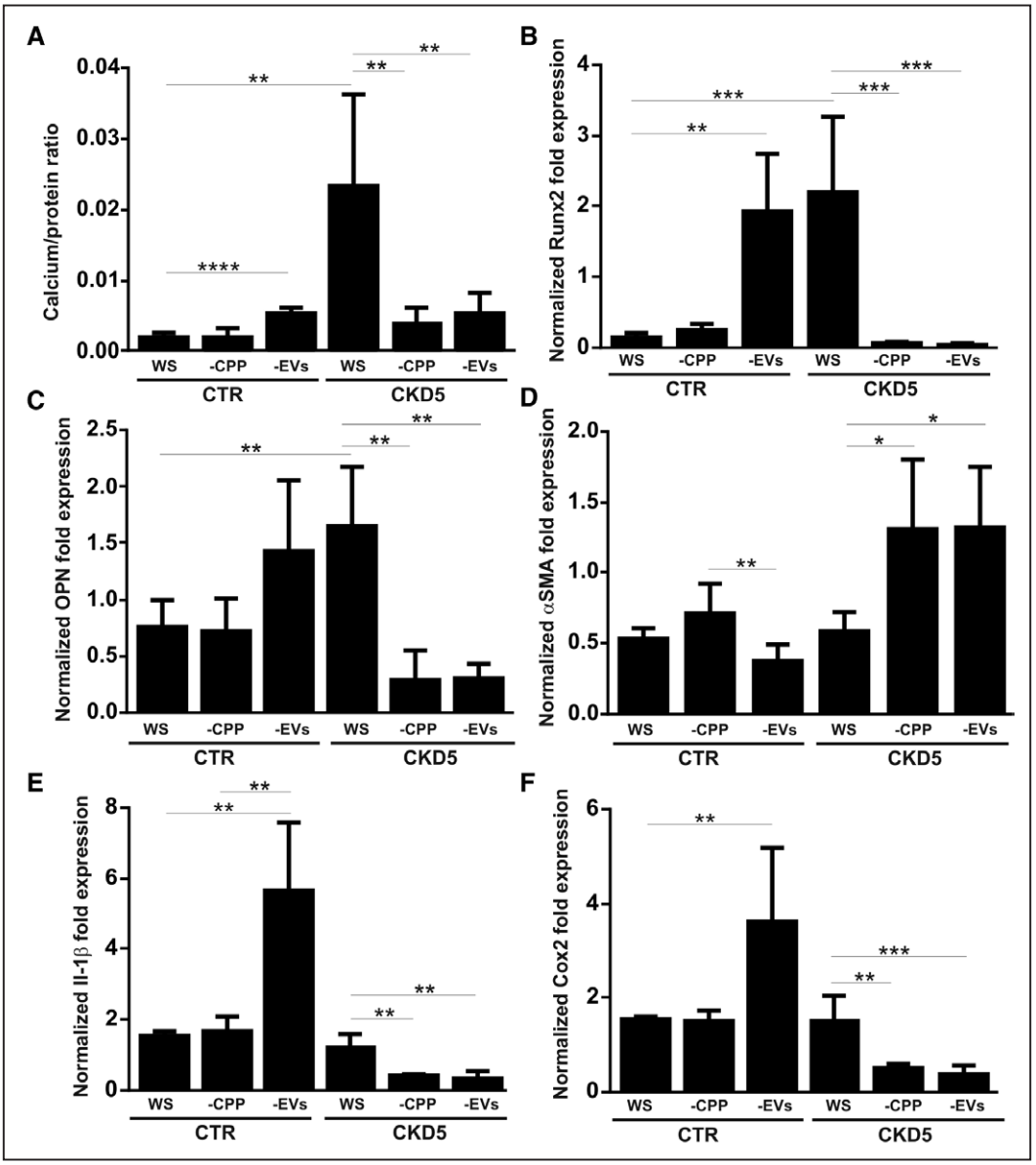

Figure 4. Removal of calciprotein particles (CPP) and extracellular vesicle (EV) particles from CKD stage 5 (CKD5) serum reduces vascular smooth muscle cell (VSMC) calcification by delaying cell differentiation and reducing inflammation. **A**, Primary VSMCs were grown in calcifying conditions for 14 d in the presence of control (CTR) and CKD5 human serum, and different variations of each: complete whole serum (WS), CPP-depleted serum (-CPP), and EV-depleted serum (-EVs). Cells were continuously pretreated with 2.5 mmol/L phosphate and then exposed to 5.4 mmol/L calcium for a period of 24 h before harvesting. The mineralization rate was determined for each condition through calcium quantification normalized to protein levels. SD was calculated (n=5) from 3 independent experiments, and 1-way ANOVA was used with multiple comparisons determined with Tukey test. Statistical significance was defined as $P \leq 0.01$ (**), $P \leq 0.001$ (***), and $P \leq 0.0001$ (****). **B–F**, Relative gene expression of osteochondrogenic differentiation (Runx2 [runt-related transcription factor 2] and OPN [osteopontin]; **B** and **C**, respectively), αSMA (α-smooth muscle actin; **D**), and inflammation (IL-1β [interleukin 1β] and cyclooxygenase-2 [Cox2]; **E** and **F**, respectively) markers was determined by quantitative polymerase chain reaction, and normalized fold expression (arbitrary units) was determined relative to zero. SD was calculated (n=5) from 3 independent experiments, and ordinary 1-way ANOVA was used with multiple comparisons determined with Tukey test. Statistical significance was defined as $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), and $P \leq 0.0001$ (****).

(interleukin 1β) and cyclooxygenase-2 (Cox2) (Figure 4E and 4F). Decreased calcification observed after CPP and EVs removal from CKD5 serum is accompanied by increased levels of αSMA (Figure 4D) and decreased levels of both osteogenic and inflammation markers Runx2, OPN, IL-1β, and Cox2 (Figure 4B, 4C, 4E, and 4F).

CPP and EV Particles Are Major Contributors for the High Calcification Potential of Uremic Serum

To further establish a link between CPPs and EVs with VSMC calcification, we evaluated the direct and independent effect of each of these nanostructures isolated from CKD5 serum as supplements to control serum. In addition, because we found reduced levels of GRP in CKD5 CPPs, we tested the effect of CPPs from CKD5 (CPP5) after incubation with cGRP. We showed that supplementation of control serum depleted from CPPs with CPP5 clearly increased VSMC calcification, whereas CPP5+cGRP rescued the CPP5-induced calcification to control levels (Figure 5A). Increased calcification induced by CPP5 is characterized by lower expression levels of the differentiation marker αSMA and the mineralization inhibitor MGP, whereas increased levels of the osteogenic markers Runx2 and OPN and inflammation markers IL-1β and Cox2 (Figure 5B). The effect of cGRP associated with CPP5 was mediated by increased levels of αSMA and MGP and decreased levels of Runx2, OPN, IL-1β, and Cox2 (Figure 5B).

Also, supplementation of control serum depleted from EVs (-EVs) with CKD5 EVs (EVs5) increased VSMCs calcification (Figure 5A). Similar to the effect observed with CPPs, increased calcification mediated by EVs5 is characterized by increased levels of the osteogenic markers Runx2 and OPN and inflammation markers IL-1β and Cox2, whereas decreased levels of αSMA (Figure 5B). Interestingly, we found a strong upregulation of MGP induced by supplementation with EVs5 (Figure 5B).

TEM imaging allowed the detection of initial and final steps of CPP5 uptake until internalization in VSMC vacuoles (Figure 5C). Uptake of EVs was analyzed by TEM and fluorescence imaging of PKH67-labeled EVs5. TEM observations showed accumulation of EVs5 in a region of VSMCs membrane invagination and vesicles inside vacuoles (Figure 5D), whereas PKH67-labeled EVs were clearly detected inside VSMCs (Figure VIII in the online-only Data Supplement).

γ-Carboxylated GRP, MGP, and Fetuin-A Constitute a Powerful Antimineralization System Efficiently Inhibiting Ca/P Mineral Formation and Maturation

To further understand the role of GRP in mineral formation and maturation, we setup in vitro assays for the formation of basic calcium phosphate (BCP), to which we added cGRP, MGP, and fetuin-A, or ucGRP (undercarboxylated GRP), MGP, and fetuin-A. Levels of mineral formation were determined by calcium quantification of the resulting mineral pellets and supernatants (Figure 6A). In control BCP assays containing only Ca and P ions, high levels of Ca in the pellets and low levels in the supernatant indicate formation of mineral crystals. However, addition of the combination of cGRP, MGP, and fetuin-A resulted in a significant reduction in calcium in the pellet of 66%, with most Ca remaining in the supernatant. Interestingly, the reduction of calcium in the pellet was not present when cGRP was substituted by ucGRP, indicating that γ-glutamate carboxylation is essential for the antimineralization function of GRP. SDS-PAGE (Figure 6B) showed that in the presence of ucGRP, fetuin-A is equally distributed between the supernatant and pellet fractions, and MGP is mostly associated with the mineral pellet. However, in the presence of cGRP, there was an increased amount of all proteins in the supernatant, particularly relevant in the case of fetuin-A, suggesting a synergistic effect to inhibit mineral formation and precipitation.

To characterize the pelleted mineral particles, their morphology was analyzed by electronic microscopy. TEM imaging revealed that the BCP control mineral pellet displayed needle-like clusters typical of CPPII particles (Figure 6C).[9,10] However, the combination of cGRP, MGP, and fetuin-A resulted in a mineral pellet characterized by distinguishable spherical aggregates resembling CPPI particles (Figure 6C), clearly indicating a delayed transformation of colloidal CPPs into crystalline mineral. Similar morphological features of cGRP, MGP, and fetuin-A pellet, with aggregates of spherical particles, was observed by scanning electron microscopy, and elemental mapping for P and Ca indicate that both elements were evenly distributed within CPPI-like particles (Figure 6D).

These results clearly indicate that γ-carboxylated GRP, MGP, and fetuin-A constitute a powerful inhibitory system to prevent crystal formation and maturation. To investigate the biological relevance of this mineralization inhibitory system, we performed coimmunoprecipitation assays in protein extracts from noncalcified aortas and isolated EVs from VSMCs cultured in control conditions. WB analysis of coimmunoprecipitation assays performed using total protein extracts from aortas captured with the CTerm-GRP antibody showed the presence of GRP, MGP, and fetuin-A (Figure 6E). Similarly, WB analysis of coimmunoprecipitation assays from VSMCs isolated EVs, which were positive for CD9 and TSG101 (Figure IX in the online-only Data Supplement), showed the presence of a protein complex containing GRP, MGP, and fetuin-A (Figure 6F).

Discussion

In this study, we provide novel mechanistic links for the prevalence of VC in CKD patients, mediated by circulating CPPs and EVs nanoparticles. Furthermore, we demonstrate that CPP particles are present in the circulation of healthy individuals differing from CKD CPPs in terms of mineralization inhibitor levels and mineral crystal maturation. We show that GRP is a constitutive component of both CPP and circulating EVs and demonstrate both in vivo and in vitro that an increase in mineral maturation is associated with deficiency of both GRP and fetuin-A. Importantly, these CPPs and EVs showing decreased levels of GRP and fetuin-A were shown to promote calcification of VSMCs by inducing osteochondrogenic differentiation and inflammation processes, strongly suggesting a pathogenic effect of CPPs and EVs from uremic serum.

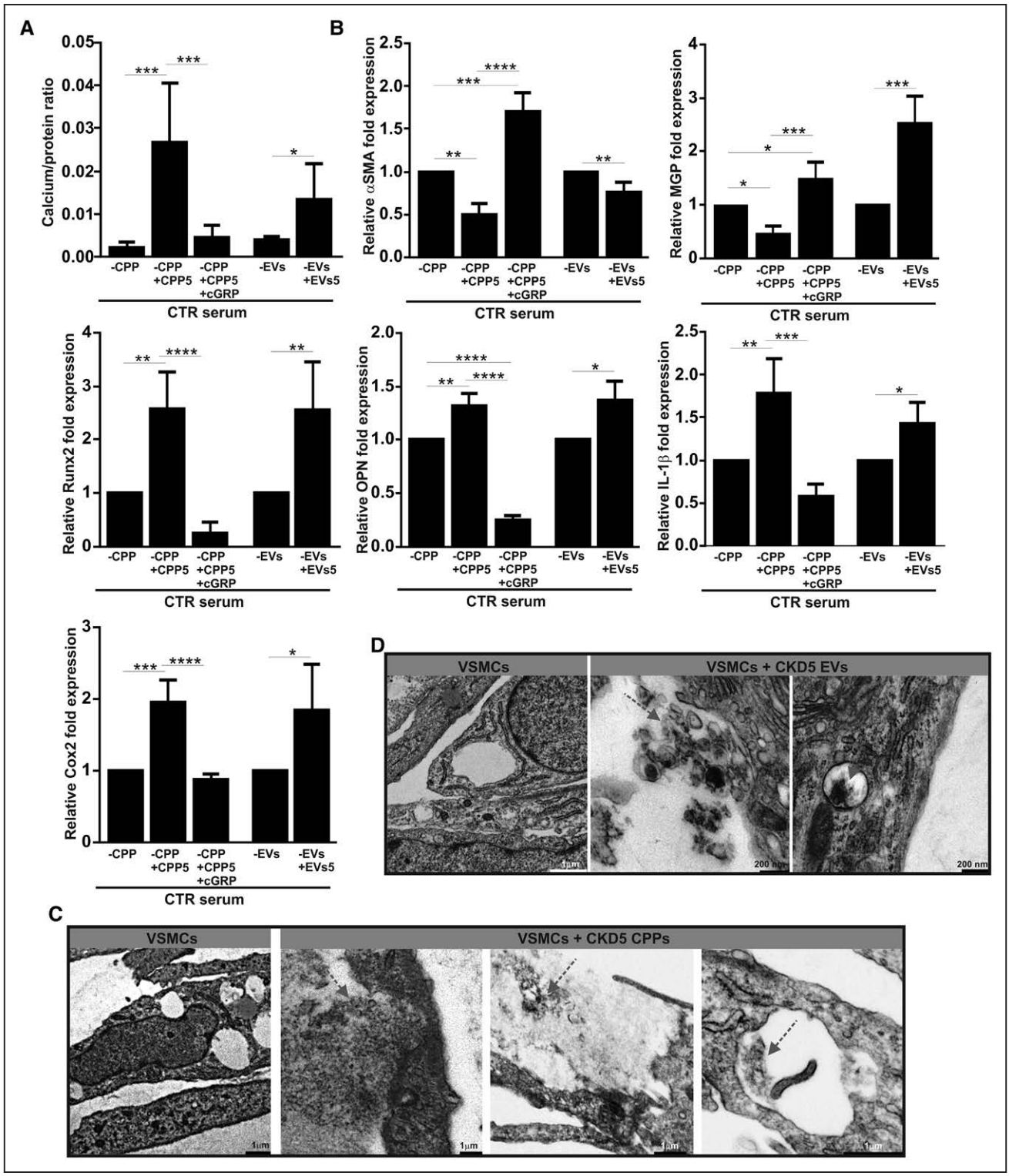

Figure 5. Chronic kidney disease stage 5 (CKD5)–derived calciprotein particles (CPP) and extracellular vesicle (EV) particles promote vascular smooth muscle cell (VSMC) calcification. **A**, Primary VSMCs were grown in calcifying conditions as described in the legend of Figure 4 in the presence of the following variations of control (CTR) human serum; CPP-depleted serum (-CPP); CPP-depleted serum supplemented with CCPs isolated from CKD5 (-CPP+CPP5); CPP-depleted serum supplemented with CCP5 incubated with cGRP (-CPP+CPP5+cGRP); EV-depleted serum (-EVs); and EV-depleted serum supplemented with EVs isolated from CKD5 (-EVs+EVs5). The mineralization rate was determined for each condition through calcium quantification normalized to protein levels. SD was calculated (n=6) from 3 independent experiments. For comparison of 3 conditions in experiments involving CPPs, 1-way ANOVA was used with multiple comparisons determined with Tukey test, and unpaired *t* test was used for comparison of 2 conditions in experiments involving EVs. Statistical significance was defined as $P \leq 0.05$ (*) and $P \leq 0.01$ (**). **B**, Relative gene expression of differentiation (αSMA [α-smooth muscle actin]), calcification inhibitor (MGP [matrix gla protein]), osteochondrogenic (Runx2 [runt-related transcription factor 2] and OPN [osteopontin]), and inflammation (IL-1β [interleukin 1β] and cyclooxygenase-2 [Cox2]) markers was determined for the conditions (*Continued*)

CPP formation has been suggested as a defense mechanism against calcium phosphate precipitation in blood.[9–11] Circulating mineral-binding proteins such as fetuin-A have a preponderant role to limit spontaneous crystal nidi formation and delay maturation into a more crystalline and insoluble hydroxyapatite-like mineral phase.[9,10] Considering that in healthy individuals serum is supersaturated with respect to Ca and P, it is likely that such a defense mechanism is constitutively functioning to maintain blood mineral homeostasis and prevent calcification. However, until now, serum CPPs have been detected in several pathological conditions but were suggested to be undetectable in healthy individuals.[9,11,12,31] A previous study, however, reported the existence of a multiprotein component containing detectable levels of Ca in CPPs isolated from control healthy subjects.[11] Our results corroborate these findings and further shown that an entity containing mineral-regulatory proteins, including fetuin-A and GRP, and a mineral phase is present in serum from both healthy and CKD individuals. CKD CPPs were found to contain decreased levels of fetuin-A and GRP and a more crystalline mineral phase with features of CPPII,[10] whereas control CPPs were characterized as CPPI particles. These CPPI can be removed from circulation through class A scavenger receptor–mediated pathways or eventually over time aggregate and develop crystalline CPPII-like structures.[10,35] Fetuin-A was shown to be predominant in CPPI and less represented in CPPII particles,[31] which is in agreement with our findings of decreased fetuin-A levels associated with CKD CPPII structures. The presence of fetuin-A is widely suggested to stabilize CPPI and retard the progression toward secondary CPPs. Nevertheless, the ability of proteins to inhibit mineral growth by binding to CaP clusters is not restricted to fetuin-A. Other proteins such as GRP are part of these CPPs playing an important role in the mineral maturation process.

At tissue level, VC in the extracellular matrix is initiated by deposition of BCP-containing EVs derived from VSMCs, forming a nidus of calcification for further mineral growth, in a process similar to bone mineralization.[4,5,18,24] Mineral nucleation sites within EVs are blocked in the presence of mineralization inhibitors such as GRP, MGP, and fetuin-A.[4,5,24] This process seems to share the same basic principles as the formation and maturation of mineral crystals in serum CPPs. We show that GRP and fetuin-A deficiency results in increased levels of mineral maturation, with increased capacity to promote VSMC calcification. Although previous studies established correlations between CPP levels and calcification in CKD[11] and several in vitro experiments showed a deleterious effect of synthetic CPPs in cultured cells by promoting inflammatory and mineralization responses,[15–18] our study demonstrates that biologically isolated CKD CPPs are pathogenic circulating entities capable of promoting VC. Possible explanations for the relation between CPP levels and calcification are suggested to be resulting from the decreased levels of free circulating fetuin-A captured by increased CPPs to prevent VC in the vascular wall[11] or the toxicity of CPP CaP nanocrystals.[9] In fact, several lines of evidence suggest that calcium and phosphate ions might not be per se the direct mediators of cellular toxicity. The real culprit might be the calcium phosphate nanocrystal product, whose formation depends on mineralization inhibitor activity. Findings supporting this notion are that synthetic CPPII, and not CPPI, induce VSMCs calcification,[13] and naked hydroxyapatite crystals have more pronounced proinflammatory effects in macrophages than synthetic fetuin-A–containing CPPs.[16,36] Moreover, serum-derived CPPs have been shown to produce a higher protective effect than synthetic CPPs in macrophage activation,[36] likely reflecting the inhibitory activity of serum components such as GRP. We have shown in vitro that γ-carboxylated GRP, fetuin-A, and MGP are able to significantly reduce BCP mineral formation and maturation and that a protein complex containing these 3 proteins exists in vivo in physiological conditions. We propose that this antimineralization system represents a powerful mechanism to regulate the dynamics of mineral formation both at systemic and tissue levels to prevent unwanted deposition of mineral. MGP is a well-established inhibitor of VC, but it is presently unclear whether MGP is involved in circulating CPPs or EVs in humans. Although the scope of this research is focused on GRP, several lines of evidence have previously suggested a close relationship between GRP, fetuin-A, and MGP. We have shown that a protein complex containing GRP, fetuin-A, and MGP is present at sites of aortic valves calcification and that GRP is involved in the mineralization competence of EVs released from VSMCs[24] as also previously reported for MGP and fetuin-A.[4,5] Moreover, a MGP–fetuin-A complex was described in chondrocyte-derived EVs and linked to increased calcification in osteoarthritis.[37] Additionally, a fetuin-A–MGP complex was identified in rat CPPs.[33] Because γ-glutamyl carboxylation is essential for the calcification inhibitory function of GRP and MGP,[24,25,38] this antimineralization system might represent a novel therapeutic target for the inhibition of ectopic calcification by functional modulation through vitamin K supplementation or by direct enrichment in protein components, such as GRP.

We have previously demonstrated that direct cGRP supplementation is able to reduce calcification of vascular tissue and articular cells and that increased levels of GRP, either exogenous or endogenous, are able to reduce proinflammatory responses of articular and immune cells.[24,25,30] This dual role of GRP as calcification inhibitor and anti-inflammatory agent is reinforced by results showing that incubation of CPPs from CKD with cGRP rescued calcification/osteogenic differentiation and inflammatory status induced in VSMCs. Indeed, CPPs have been associated with increased serum inflammatory markers[39] and inducing

Figure 5 Continued. described in the graph by quantitative polymerase chain reaction, and relative fold expression was determined relative to the respective control condition; CTR CPP depleted serum (-CPP); and CTR EVs depleted serum (-EVs). SD was calculated (n=4) from 2 independent experiments, and 1-way ANOVA was used with multiple comparisons determined with Tukey test. Statistical significance was defined as $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq P \leq 0.001$ (***), and $P \leq 0.0001$ (****). **C** and **D**, Transmission electron microscopy imaging of VSMCs exposed to CKD5 CPPs for 12 h (**C**) and to CKD5 EVs for 3 h (**D**), showing the process of nanostructures internalization. Red arrows indicate the supplemented material, CPPs (**C**) and EVs (**D**).

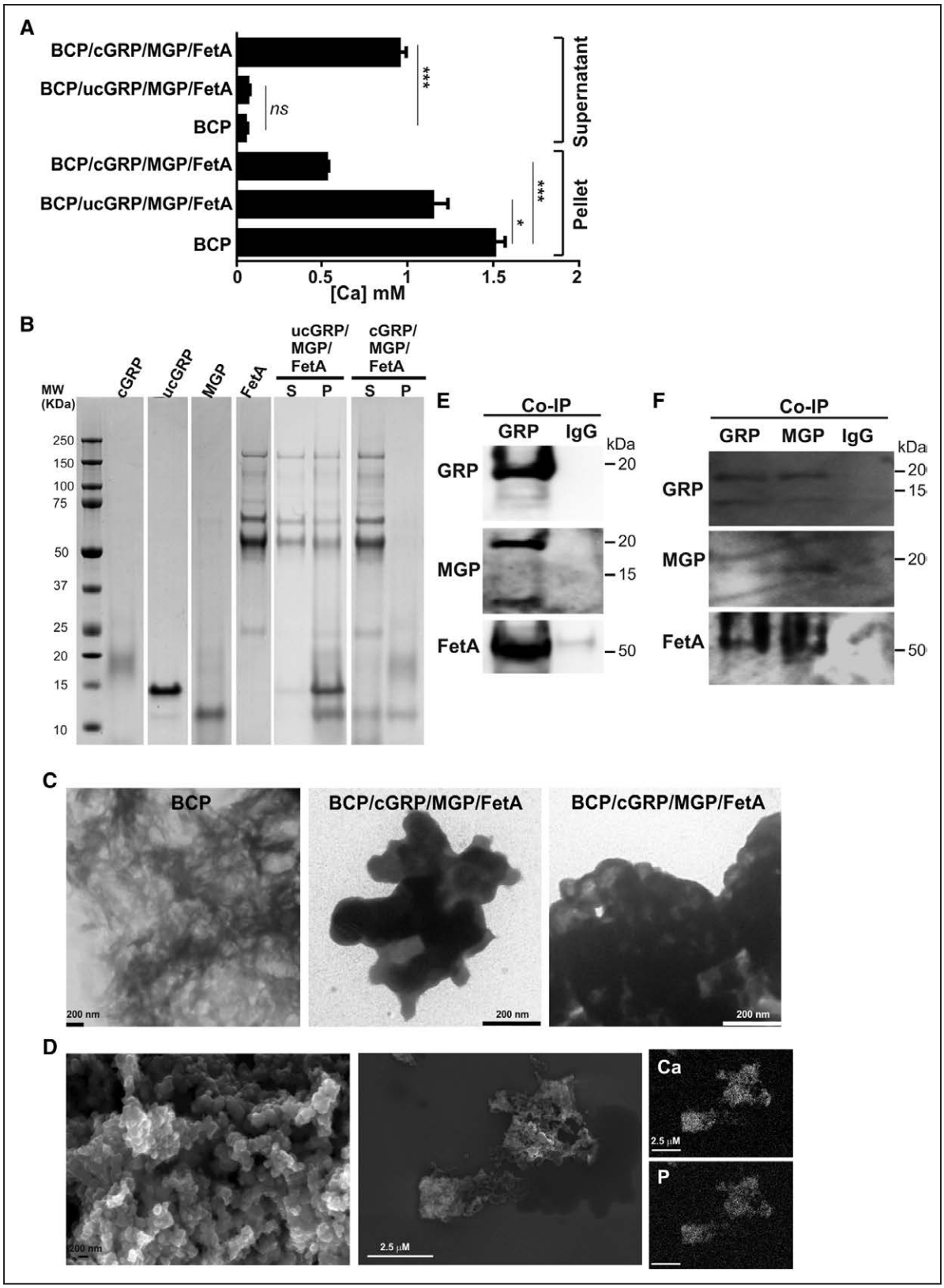

Figure 6. γ-Carboxylated GRP (Gla-rich protein), MGP (matrix gla protein), and fetuin-A constitute a powerful mineral formation and maturation inhibitory system. **A**, Calcium quantification of the resulting pellets and supernatants from in vitro assays for the formation of basic calcium phosphate (BCP) crystals, in the absence of proteins (BCP), in the presence of cGRP, fetuin-A and MGP (BCP/cGRP/fetuin-A/MGP), or ucGRP, fetuin-A, and MGP (BCP/ucGRP/fetuin-A/MGP). SD was calculated from 3 independent experiments, and 1-way ANOVA was used with multiple comparisons determined with Tukey test. Statistical significance was defined as $P \leq 0.05$ (*) and $P \leq 0.01$ (**). **B**, SDS-PAGE and coomassie brilliant blue staining of the cGRP, ucGRP, MGP, and fetuin-A proteins in 25 mmol/L boric acid pH 7.4, used in (*Continued*)

proinflammatory cytokine release and reactive oxygen species, although to a less extent than naked HA crystals.[36] So, in addition to the well-known function of fetuin-A as a strong anti-inflammatory agent,[40] our results indicate that GRP has an active role in protecting the vasculature against calcification and inflammation induced by CPPs with strong implications for calcification/inflammation-related diseases such as CKD and atherosclerosis.

In addition to CPPs, we also found that circulating EVs of CKD5 contained decreased levels of fetuin-A and GRP and thereby promote VSMC calcification, through increased osteochondrogenic differentiation and inflammation. Interestingly, these EVs also contained higher levels of GRP and MGP mRNA. Although the biological significance of such finding is presently unclear, it is known that virtually all cells produce and release EVs that can reach circulation.[17,22] It has been suggested that the mechanism of EVs loading is a regulated process that might not entirely reflect the intracellular environment.[41] In this study, EVs were isolated from serum representing a heterogeneous mixture in terms of cell source. Lower levels of GRP and fetuin-A in these complex mixtures of EVs might reflect an overall deficiency in mineralization inhibitors associated with CKD or an altered mechanism of endosomal protein trafficking, as suggested by the decreased levels of TSG101 in CKD5 EVs. TSG101 is a subunit of the endosomal sorting complex required for transport-1, and endosomal sorting complex required for transport complex deficiency can interfere with the regulation of cargo sorting into EVs, with consequent dysregulation of cell-signaling processes.[41,42]

The role of GRP and fetuin-A as inhibitors of mineral crystal nucleation/maturation has been previously established and associated with the calcification potential of VSMCs EVs.[4,5,24] Our results support the need for further elucidation of the mechanisms mediating the effect of circulating CPPs and EVs in VC. We already showed that this is an active cell-mediated process, where VSMCs can engulf both CPPs and EVs, in concordance with previous work showing the uptake of synthetic CPPs and EVs.[13,15,36] TEM imaging analysis indicates that these nanostructures are incorporated by VSMCs through endocytosis. Interestingly, although calcification-prone CPPs and EVs induce VSMCs osteochondrogenic differentiation and proinflammatory responses, we suggest that they might also function as transport mechanisms to deliver circulating mineralization inhibitors into VSMCs in physiological conditions. Our results show that removal of EVs from control serum loaded with GRP and fetuin-A enhanced VC, suggesting a physiological function on the protection of vascular health. Considering the current knowledge in the EV field regarding their capacity to transfer molecular cargo to target cells, it is likely that circulating EVs that are taken up by VSMCs contribute to modulate vascular homeostasis through the release of active biomolecules. Interestingly, MGP expression was found upregulated in VSMCs treated with CKD5 EVs containing high levels of MGP mRNA. Also, we found the complete GRP open-reading frame in control isolated EVs, suggesting that GRP can potentially be translated in VSMCs from circulating EV mRNA. Previously it was shown that fetuin-A is taken up from the circulation into VSMCs through uncertain mechanisms.[4] Our data represents, at least in part, a mechanism for fetuin-A loading into VSMCs. However, more work is needed to reveal whether EV-mediated uptake of GRP and fetuin-A by VSMC represent a calcification inhibitory mechanism acting in vivo. Also, efforts should be undertaken to understand the specific cell(s) origin of circulating EVs containing GRP and fetuin-A. In addition to VSMCs, we have previously reported that GRP is also present in macrophage-derived EVs in vitro,[30] and it is likely that other GRP-expressing cells release GRP-loaded EVs. Although many questions are still open, these data represent a high impact on the current view of intercellular signaling with possible implications on the mode of action of GRP as a systemic calcification inhibitor and anti-inflammatory agent. Our results show that both CPPs and EVs can modulate osteochondrogenic differentiation and inflammatory processes in VSMCs. Because calcification and inflammation are interconnected processes contributing for atherosclerosis development, which is often present in CKD patients, this study should be further performed using more complex and closer-to-in vivo model system that will clarify the overall impact of uremic serum in CKD.

In conclusion, circulating CPPs and EVs are determinants of VC in CKD, with the capacity to modulate VSMC responses through a differentiation and inflammation stress condition, leading to increased mineral deposition. In this process, decreased levels of GRP and fetuin-A promote CPPs and EVs pathogenicity. Additional efforts are required to understand a possible relation between loading of circulating EVs and VC stress in CKD, but from a biomarker perspective, our results foresee high potential for the use of EVs and CPPs in CKD diagnostic, particularly through their GRP and fetuin-A content. Possible approaches targeting the increase of γ-carboxylated GRP bioavailability might represent promising therapeutic approaches for VC-related diseases.

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Figure 6 Continued. the assays described in (**A**), and the resulting supernatant (S) and pellet (P) fractions after in vitro assays for the formation of BCP in the presence ucGRP, MGP, and fetuin-A, and cGRP, MGP, and fetuin-A. Relevant molecular mass markers (kDa) are indicated. **C**, Pellets from BCP and BCP/cGRP/fetuin-A/MGP assays were stained with 1% aqueous uranyl acetate and analyzed by transmission electron microscopy. **D**, Pellets from BCP/cGRP/fetuin-A/MGP assays were analyzed by scanning electron microscopy coupled with energy-dispersive x-ray spectroscopy elemental mapping for calcium (Ca) and phosphorous (P). **E** and **F**, Western blotting with CTerm-GRP, MGP, and fetuin-A antibodies (indicated on the left side), of eluted proteins obtained after Co-coimmunoprecipitation reactions with either CTerm-GRP and IgG antibodies using noncalcified aorta tissue protein extracts (**E**), or CTerm-GRP, MGP, and IgG antibodies (Co-IP-GRP, Co-IP-MGP, Co-IP-IgG) using protein extracts of EVs isolated from vascular smooth muscle cells cultured in control conditions (**F**). Relevant molecular mass markers (kDa) are shown on the right side of **E** and **F**.

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Disclosures

The tools and methods described in this manuscript are included in a PCT patent application PCT/PT2009000046 and exclusive rights are licensed to Genogla Diagnostics. Dr Simes and Dr Viegas are cofounders of GenoGla Diagnostics; Dr Vermeer is founder of VitaK. The other authors report no conflicts.

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Highlights

- GRP (Gla-rich protein) is a constitutive component of circulating calciprotein particles and extracellular vesicles.
- In vivo, increased mineral maturation in calciprotein particles from chronic kidney disease stage 5 patients is associated with lower levels of mineralization inhibitors GRP and fetuin-A, and in vitro, γ-carboxylated GRP, MGP (matrix gla protein), and fetuin-A constitute a powerful inhibitory system for crystal formation and maturation.
- A protein complex containing GRP, MGP, and fetuin-A was identified in blood vessels and vascular smooth muscle cells released extracellular vesicles, likely representing a constitutive physiological calcification inhibitory system.
- Calciprotein particles and extracellular vesicles from chronic kidney disease stage 5 patients with lower GRP and fetuin-A promote calcification of vascular smooth muscle cells in an active cell-mediated process involving the uptake of these nanostructures and reprogramming of vascular smooth muscle cells by inducing osteochondrogenic differentiation and inflammation processes.
- Incubation of γ-carboxylated GRP with uremic calciprotein particles rescues calcification by retaining the vascular smooth muscle cell contractile phenotype.