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Abstract

Objective-The drug warfarin blocks carboxylation of vitamin K dependent proteins and acts as an anticoagulant and an accelerant of vascular calcification. The calcification inhibitor matrix Gla protein (MGP), produced by vascular smooth muscle cells (VSMCs), is a key target of warfarin action in promoting calcification, however it remains unclear whether proteins in the coagulation cascade also play a role in calcification.

Approach and Results-Vascular calcification is initiated by exosomes and proteomic analysis revealed that VSMC exosomes are loaded with Gla-containing coagulation factors; IX and X, prothrombin (PT) and proteins C and S. Tracing of Alexa488-labeled PT showed that exosome loading occurs by direct binding to externalized phosphatidylserine (PS) on the exosomal surface and by endocytosis and recycling via late endosomes/multivesicular bodies (LE/MVB). Notably, the PT Gla domain and a synthetic Gla-domain peptide inhibited exosome-mediated VSMC calcification by preventing nucleation site formation on the exosomal surface. PT was deposited in the calcified vasculature and there was a negative correlation between vascular calcification and the levels of circulating PT. In addition, we found that VSMC exosomes induced thrombogenesis in a tissue factor (TF)- and PS-dependent manner.

Conclusions- Gamma carboxlyated coagulation proteins are potent inhibitors of vascular calcification suggesting warfarin action on these factors also contributes to accelerated calcification in patients receiving this drug. VSMC exosomes link calcification and coagulation acting as novel activators of the extrinsic coagulation pathway and inducers of calcification in the absence of Gla-containing inhibitors.

Graphic Abstract

Abbreviations

EV, extracellular vesicles

F1, prothrombin fragment 1

F1.2, prothrombin fragment 1+2

Gla, Carboxyglutamic acid

LE/MVB, late endosomes/multivesicular bodies

MGP, matrix Gla protein

PIVKA-II, protein induced by vitamin K absence or antagonism II

Pre2, prethrombin-2 (Pre-2)

PS, phosphatidylserine

PT, prothrombin

TF, tissue factor

VSMCs, vascular smooth muscle cells (VSMCs),

Introduction

Vascular calcification contributes to increased cardiovascular morbidity and mortality in ageing, atherosclerosis, diabetes mellitus and chronic kidney failure[1, 2]. It occurs in the medial and intimal layers of arteries and is driven by vascular smooth muscle cells (VSMCs) which can undergo osteo-chondrogenic differentiation and secrete calcifying exosomes or apoptotic bodies that initiate deposition of calcium (Ca) phosphate (P) crystals[3-5].

Under physiological conditions VSMCs are protected from calcification by expression of inhibitors including the vitamin K-dependent proteins; matrix Gla protein (MGP), bone Gla protein (BGP; osteocalcin), Gla rich protein (GRP) and growth arrest-specific gene-6 protein (Gas 6)[6-9]. These proteins all contain specific glutamate residues (Glu) necessary for their function that are carboxylated in a vitamin K-dependent manner. Increased accumulation of uncarboxylated, inactive forms of MGP, GRP and BGP have been observed in the calcified vasculature or the circulation of patients with vascular calcification stressing the importance of adequate vitamin K-status in the inhibition of calcification[8, 10-12].

It is still unknown how calcification is inhibited by vitamin K-dependent proteins. Reduced Gas6 carboxylation results in inhibition of Axl-dependent anti-apoptotic pathways and subsequent VSMC apoptosis and calcification[9, 13]. MGP has been implicated in BMP-2 binding and inhibition of BMP2-dependent osteogenic signalling[14]. MGP has also been detected in VSMC exosomes where it inhibits Ca/P precipitation although the precise mechanisms by which the Gla domain inhibits the nucleation of Ca salts remain unclear[15, 16]. Interestingly, VSMCs internalise liver derived fetuin-A from the circulation and load it into exosomes where it acts as a potent inhibitor of calcification. Thus, both locally expressed as well as serum-derived proteins are involved in the prevention of exosome-mediated VSMC calcification[5].

Circulating vitamin K-dependent coagulation factors including prothrombin (PT) and factors VII, IX and X and proteins C and S also harbour Gla residues which mediate Ca-dependent protein binding to negatively charged phospholipids, such as PS [17-21]. Activation of coagulation in the vasculature has been linked to PS-exposing extracellular vesicles (EVs) shed by apoptotic macrophages, endothelial cells, erythrocytes and VSMCs[17, 22 23, 24]. In particular, the coagulation cascade is triggered by VSMC EVs bearing tissue factor (TF) that can activate Factor VII with subsequent activation of the prothrombinase complex (consisting of Factor Va, Factor Xa and calcium) to catalyze PT conversion to thrombin[17, 22, 25-28]. Anticoagulation therapy with warfarin causes elevation of circulating levels of liver-derived uncarboxylated PT (PIVKA-II) and results in the inhibition of thrombogenesis[29]. Besides its effect on vitamin K-dependent coagulation proteins, warfarin increases circulating levels of undercarboxylated MGP and BGP

and causes vascular calcification in rodent models and accelerates both valve and vascular calcification in man[30-33]. However, despite the global use of anticoagulation therapies in clinical practice, the effects of warfarin on VSMC mediated calcification remain elusive.

Here we report that circulating vitamin K-dependent coagulation proteins, PT in particular, can bind to the surface of VSMC exosomes via PS and can also be loaded into exosomes by cellular internalization and recycling via the late endosome/multivesicular body (LE/MVB) compartment. We identify PT as a novel circulating calcification inhibitor that also acts to induce thrombin generation in a TF- and PS-dependent manner on the surface of exosomes. Thus, anticoagulation therapy with warfarin may enhance vascular calcification via impaired carboxylation of vitamin K-dependent coagulation factors delivered to VSMC exosomes, which play a dual role in calcification and coagulation.

Material and methods

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

Results

Circulating Gla proteins are abundant in VSMC exosomes

We previously demonstrated the recycling of the circulating serum protein, fetuin-A by VSMC exosomes.[5] To determine whether other serum proteins are recycled in VSMC exosomes we interrogated a proteomic dataset of human VSMC-derived exosomes[5], isolated under normal or calcifying conditions, for bovine proteins indicative of serum uptake. The most abundant bovine proteins in VSMC exosomes were albumin, fetuin-A and fibronectin (Table 1). Notably, circulating vitamin K-dependent coagulation proteins including factors II (PT), IX and X, and anticoagulation proteins C and S were also detected in VSMC-derived exosomes and their loading was increased under calcifying conditions (Table 1, *Number of Assigned Spectra*).

We compared the primary structure of circulating vitamin K-dependent coagulation proteins and MGP[7]. The alignment of the Gla-domain sequences revealed a similarity, of over 50% in the composition of the Gla-domain between vitamin K-dependent coagulation proteins (Supplementary Figs. S1A and S1B). Prominently, there were 4 conserved Glu-residues and one cysteine residue in all these proteins suggesting coagulation proteins may potentially modulate vascular calcification.

Gla proteins differentially inhibit VSMC calcification in vitro

VSMCs were treated with physiological doses of a number of Gla containing proteins to test their capacity to inhibit calcification *in vitro* (Fig. 1A). MGP is a potent local inhibitor of calcification however circulating MGP has no inhibitory properties *in vivo*[34] and is only effective *in vitro* at doses above physiological levels[35]. As expected MGP1-53 (Supplementary Figs. S2A and C), osteopontin and osteocalcin had little effect on calcification when added exogenously to the media at circulating concentrations (Fig. 1A). In contrast PT, the most abundant circulating coagulation factor with physiological concentrations ~1.4μM was able to potently inhibit calcification in a manner similar to fetuin A (Fig. 1A).

PT expression was not observed in VSMCs either by western blotting or PCR (Supplementary Fig. S3A). However, incubation of VSMCs with PT resulted in its accumulation in exosomes, apoptotic bodies and whole cell lysates (Fig. 1B) with exosomes also enriched with PT intermediate activation products, F1 and F1.2 (Fig. 1B). In agreement with our proteomic data, calcifying conditions increased the levels of PT and F1 both in exosomes and VSMCs (Fig. 1C).

PT inhibits exosome-induced calcification and reduces VSMC apoptosis.

To test further the effects of PT on vascular calcification we treated VSMCs in elevated Ca/P conditions and assessed calcification by ^{45}Ca incorporation and Alizarin red S staining (Figs. 2A and B). PT inhibited calcification in a dose-dependent manner at concentrations within the physiological range. The inhibitory effects of PT were more pronounced in Ca- rather than P-induced calcification (Supplementary Figs. S4A and S4B). Fetuin-A blocks calcification by binding to nascent Ca/P crystals preventing further growth and acting as a "mineral chaperone".[36, 37] Fetuin-A and PT inhibited VSMC calcification in a synergistic manner indicating that PT acts via different mechanisms (Fig. 2C) and in agreement with this fetuin-A but not PT completely prevented Ca/P precipitation *in vitro* (Fig. 2D).

To study the effects of PT on exosome-initiated calcification, exosomes were isolated from VSMCs treated in the absence or presence of PT and their calcification potential was quantified *ex vivo*[15]. Exosomes isolated from Ca/P treated VSMCs displayed calcification (Fig. 2E). Direct addition of PT to exosomes isolated from VSMCs incubated in Ca/P media significantly reduced exosome calcification (Fig. 2E), suggesting PT may inhibit calcification by binding to PS externalized on the exosome surface thus preventing nucleation site formation. However, inhibition of exosome calcification was also observed when VSMCs were treated with PT for 24h and then washed to remove PT from the media before exosome isolation 24h later, suggesting PT is also loaded into exosomes via the LE/MVB pathway.

VSMC apoptosis also contributes to calcification and treatment of VSMCs with calcifying media resulted in apoptosis of approximately 20-25% of cells as assessed by TUNEL staining and time-lapse microscopy (Supplementary Figs. S4C and S4D). Addition of PT significantly reduced VSMC apoptosis induced by elevated Ca/P, but had no effect on apoptosis induced by serum withdrawal (data not shown), suggesting PT can prevent mineral overload induced apoptosis.

PT is recycled in VSMC exosomes in a Ca-dependent manner.

To test if PT can directly bind to VSMC exosomes we immobilised exosomes on latex beads, incubated with Alexa488-labelled PT and observed strong binding (Fig. 3A). The interaction of PT with PS is mediated by the N-terminal F1 domain harbouring Gla-residues[20] and, consistently, addition of annexin A5 or F1 blocked PT binding to exosomes (Fig. 3A). We also observed reciprocal competition for interactions with exosomal PS between PT and annexin A5 (Supplementary Fig. S3B).

Addition of alexa488-labelled PT to VSMCs resulted in endocytosis of full-length PT in a Ca-dependent manner (Fig. 3B). To test whether PT endocytosis is mediated by high-affinity

binding to a cellular receptor or fluid-phase uptake[38], we studied endocytosis of alexa488-labelled PT in the presence of unlabeled PT. Notably, endocytosis of alexa488-labelled PT was inhibited by 5-fold molar excess of unlabelled PT, indicating that uptake is mediated by high affinity binding of PT to the cell surface potentially via Gla/PS binding (Fig. 3C). Colocalization with EEA1 and CD63 showed that PT is endocytosed by VSMCs and delivered to early endosomal and LE/MVB compartments (Fig. 3D). Immunocytochemistry also confirmed that PT-Alexa488 uptake was further stimulated by elevated Ca and inhibited by unlabelled PT (Fig. 3D). To distinguish PT which binds to exosomes in the cell media, and PT which is endocytosed and recycled in exosomes via LE/MVB we incubated VSMCs with PT for 16h ("pulse") and then washed cells with acidic buffer (pH 2.5) to remove all cell-surface bound PT and continued incubation in media without PT ("chase"). Exosomes isolated from VSMCs incubated in the presence of PT in the media, were enriched with full-length protein and fragments, F1.2 and F1 (Fig. 3E). In contrast, exosomes obtained after the chase treatment contained full length PT and its intermediate activation product with molecular weight ~34kDa, most likely Pre2, an inactive α-thrombin precursor consisting of the catalytic domain. (Fig. 3E and Supplementary Fig. S1C). Thus, PT is loaded on, and in, exosomes by direct surface binding and cellular endocytosis.

The Gla domain but not proteolytic activity is required for VSMC calcification inhibition by PT.

To identify the domain of PT responsible for blocking calcification we tested different PT forms (Fig. 4A). F1 which contains 10 Gla residues was the most potent inhibitor, even more effective than full length PT. F2, which does not contain a Gla domain, and uncarboxylated (Glu)-PT were also able to inhibit calcification although to a much lesser extent, indicating that several domains contribute to PT effects on calcification. In contrast, thrombin had no effect on VSMC calcification (Fig. 4A). To confirm that catalytic activity is not required for the PT inhibitory effect, we showed that addition of the thrombin inhibitor hirudin did not abrogate PT inhibitory effects on VSMC calcification (Fig. 4B). Finally, to investigate whether other Gla-containing coagulation proteins identified in exosomes may also affect VSMC calcification, we used a Gla peptide representing the N-terminal region of protein S containing 11 Gla residues which forms a stable conformation due to its intramodular disulfide loop (Supplementary Figs. S2B and S2C). Addition of this Gla peptide completely abrogated VSMC calcification (Fig. 4B) confirming the utility of Gla residues in inhibiting calcification.

PT accumulates at sites of calcification in vivo and is reduced in patients with vascular calcification.

To confirm a relationship between calcification and PT we analysed the distribution of PT in the calcified vasculature using an F1-specific antibody. Immunohistochemistry showed that low levels of PT were deposited in the healthy vessel wall while in calcified vessels PT was significantly present in both intimal and medial layers (Fig. 4C). Moreover, we observed overlap between the distribution of PT and fetuin-A, suggesting that PT is deposited in the vessel wall most likely via exosomes in a manner similar to fetuin-A[5, 36].

Fetuin-A levels are reduced in patients with vascular calcification, thus we tested whether there is also a correlation between vascular calcification and PT concentration. Vascular calcification of the iliac arteries was assessed by plain X-ray of the pelvis (Table 2). Patients with vascular calcification had lower levels of PT ($p<0.01$) when compared to patients without calcification. Univariate logistic regression confirmed this finding ($p=0.012$; hazard ratio 0.971; 95% confidence interval 0.948–0.993) and after adjustment for age, gender, and diabetes mellitus the finding remained significant ($p=0.004$; hazard ratio 0.905; 95% confidence interval 0.845–0.969). Addition of fetuin-A levels to the multivariable model did not change the results (data not shown).

VSMC exosomes display TF and PS-dependent procoagulant activity.

The accumulation of PT activation products, F1 and F1.2 in exosomes (Fig. 1B) suggested that exosomes may play a role in thrombin activation (Supplementary Fig. S1C) so we tested if VSMC exosomes contain TF and found it was enriched in exosomes but not apoptotic bodies (Fig. 5A). The addition of VSMC exosomes to plasma potently stimulated thrombogenesis, which was abrogated in the presence of annexin A5 (Fig. 5B) confirming thrombogenic activity is via PS exposure. Once PT is activated, F1 and F1.2 bound to exosomes may prevent further activation of PT by blocking PS sites. Indeed, exosomes isolated from VSMCs pre-incubated with PT demonstrated reduced thrombogenic activity (Fig. 5C).

Notably, no thrombin activation was observed in PT-deficient plasma upon addition of exosomes with bound PT (data not shown). PT binding to exosomes may prevent prothrombinase formation by blocking PS, so we tested if exosome-associated PT can be activated by a pre-activated prothrombinase complex. First, we incubated PT and factors Va/Xa with exosomes isolated from VSMCs incubated in the absence of PT and analysed the supernatant and exosome-associated products by western blotting using antibodies specific for either F1 or catalytic domain (Fig. 5D). No PT was detected in the exosomes isolated from VSMCs incubated in PT-free conditions (Fig. 5D, *lane 1*). Upon addition of PT we observed soluble and exosome-associated full-length PT (Fig. 5D, l*ane 2*). Addition of activated

prothrombinase (Va/Xa) along with PT resulted in the release of thrombin heavy chain (B chain) with molecular weight ~31kDa in the supernatant phase (Fig 5D, *lane 3*). We also observed B chain and activation product F1 associated with exosomes (Fig 5D, *lane 3*). Next, we analysed exosomes isolated from VSMCs treated in the presence of PT. Using anti-F1 antibodies we detected PT and PT intermediate activation products, F1, F1.2 associated with exosomes (Fig. 5D, *lane 4*). Using an anti-catalytic domain antibody we detected PT and an intermediate activation product with molecular weight ~34kDa (Fig. 5C, *line 4*), again most likely Pre2 as compared to the position of B chain (Fig 5D, *lane 3*). Addition of prothrombinase had no effect on exosome-associated PT and PT activation products (Fig. 5D, *line 5*), indicating that there is a pool of exosome-bound PT and PT intermediate activation products not available to prothrombinase. Thus, VSMC exosomes are thrombogenic however accumulation of PT and PT intermediate activation products reduces their pro-thrombogenic properties in a negative-feedback loop.

Altogether these data show that VSMC exosomes activate coagulation in a TF- and PS-dependent manner and accumulation of PT and PT activation products diminish both PS-mediated pro-calcific and pro-coagulant exosomal activities in a negative-feedback manner ~~(Fig. 6)~~.

Discussion

PT is loaded on VSMC exosomes and protects against calcification in a Gla-dependant manner.

Vitamin K-antagonist treatment aimed at the prevention of thromboembolic complications causes accelerated vascular calcification in both animal models and patients[32, 33] and this effect is thought to be associated with accumulation of uncarboxylated endogenously expressed Gla proteins such as MGP and GRP[7, 8, 10, 30]. In the present study we show for the first time that the Gla-residues in circulating coagulation factors, PT in particular, also contribute to inhibition of vascular calcification. As opposed to fetuin-A, a circulating calcification inhibitor which acts by direct binding to nascent growing calcium phosphate crystals to limit growth[5, 37], PT inhibits exosome-mediated calcification via a PS/Gla interaction, thus disrupting nucleation site formation. ‘Pulse-chase” experiments indicated that there are two mechanisms of PT loading into exosomes – direct binding to the exosomal membrane and calcium-dependent endocytosis and secretion via the LE/MVB/exosome pathway. The contribution of the latter pathway of PT loading into exosomes on calcification is currently unclear. This PT pool may be loaded inside

exosomes via unknown mechanisms described previously for fetuin-A[5, 39] where it can shield luminal PS thus enhancing the overall protective effects against calcification. Interestingly, PT endocytosis and exosomal loading were stimulated by calcium, which is required for the PS/Gla interaction[40] and elevated calcium conditions have been observed in atherosclerotic plaques[41] and in CKD patients suggesting that the internal loading pathway may be important under conditions of mineral imbalance. Accumulating evidence demonstrates the toxicity of crystalline Ca/P particles thus inhibition of crystal nucleation by PS/Gla interactions could also limit VSMC apoptosis[42, 43]. Interestingly, fragment F2 also displayed a modest protective effect indicating that the F2 kringle 2 domain could contribute to the regulation of VSMC viability in calcifying conditions by modulating cell signalling but this hypothesis is yet to be tested.

Importantly, extensive calcification of elastic fibres is commonly observed in patients with pseudoxanthoma elasticum harbouring mutations in γ-glutamyl carboxylase which causes multiple coagulation factor deficiency[44] providing further evidence that Gla-containing coagulation factors may regulate vascular calcification *in vivo.* PTs role in calcification is also supported independently by previous reports showing its presence in association with calcified kidney stones[45]. In this study we also detected PT in close association with calcified regions in human arteries where its distribution overlapped with fetuin-A deposits and the reduction of circulating PT levels negatively correlated with vascular calcification. These data mirror previous clinical data with fetuin-A showing reduced levels in dialysis patients who exhibit enhanced vascular calcification and mineral imbalance[46]. Interestingly, as opposed to MGP clinical data, the appearance of minute levels of under-carboxylated PT in the circulation (>2ng/ml which corresponds to ≈0.00003μmol/L) is not associated with vascular calcification[47] as long as the total circulating PT levels remain high (≈1.4μmol/L). Although the mechanisms of fetuin-A and PT loss in the circulation in dialysis patients are yet to be investigated it is tempting to speculate that the major cause is the formation of fetuin-mineral complexes consisting of Ca/P and fetuin-A initially identified in the serum of rats treated with bisphosphonates[48]. Formation of complexes between fetuin-A and nascent mineral nuclei into primary calciprotein complexes is thought to inhibit further mineral growth and may facilitate clearance from the blood[48] and these complexes have been recently associated with coronary artery calcification scores and aortic aortic pulse wave velocity in CKD patients[49, 50]. However, the exact composition of these complexes remains elusive and PT and fetuin-A-loaded exosomes may contribute by providing the membrane surface to form the fetuin-A-mineral complex core at early phases of calciprotein complex formation. *In vivo* these complexes are yet to be tested for the presence of PT and exosomal

markers such as CD63 or Tsg101 and it would also be interesting to test how warfarin treatment affects the composition of these complexes.

VSMC exosomes are thrombogenic

Recent studies suggest that thrombogenic activity in the atherosclerotic plaque is associated with EVs shed by apoptotic cells, especially VSMCs.[17, 22, 25, 26] Although VSMC EV thrombogenic activity exceeds the potency of platelet-derived EVs, most likely due to the presence of TF and PS, the mechanisms of thrombogenic EV secretion by VSMCs remained unknown[22]. TF has recently been shown to be secreted in exosomes in various cell types and was detected in VSMCs in cytoplasmic MVB-like structures and in VSMC culture media in small particles of an unknown nature[24, 51, 52]. Here we report that apart from triggering calcification, PS externalized on VSMC exosomes, acts as a catalytic surface for thrombogenesis. Interestingly, aactivation of PT on apoptotic cells or synthetic PS/PC vesicles occurs via the initial cleavage of PT at R320 yielding meizothrombin, a potent activator of anti-coagulant pathways[53, 54]. In contrast, we detected Pre2 and F1.2 on VSMC exosomes indicating that activation of PT on VSMC exosomes occurs by the PT cleavage at R271 generating catalytically inactive Pre2 and F1.2 and Pre2 can be instantly cleaved at R320 generating procoagulant α-thrombin[55]. This specificity may be defined by specific membrane proteins, such as integrins as reported earlier for platelets[56]. Unexpectedly, we observed a pool of exosome-associated PT and Pre2 that was not available for activation by factor Xa and these exosomes displayed reduced thrombogenic activity. There are two possible explanations for this pool of PT that cannot be activated. Firstly, PT may be loaded inside the exosomal lumen as discussed above. Secondly, activation of PT by factor Xa, by cleavage of the R320 bond in particular, requires membrane binding of factor Xa suggesting sterical hindrance may prevent activation of PT and Pre2[28].

In conclusion, PT is a novel circulating vascular calcification inhibitor acting via Gla/PS binding to exosomes. Binding of PT to exosomes also activates coagulation pathways whilst gradual loading of exosomes with PT and PT activation products reduces both pro-calcification and pro-coagulant activities by Gla/PS interactions. Hence, vascular protection in patients at high risk for vascular calcification may be achieved by using direct oral anticoagulants instead of warfarin or by supplementation with small Gla peptides e.g. Gla peptide derivative from protein S. Modulation of exosome biogenesis pathways, such as SMPD3 which is activated in vascular pathologies[57, 58] may also provide alternative therapeutic approaches to reduce plaque thrombogenicity causing late stage thrombotic events in patients after myocardial infarction or coronary artery stenting[59].

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Conflict of Interest Disclosures

None.

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Highlights.

1. Circulating Gla proteins are abundant in VSMC exosomes.
2. PT is a novel circulating vascular calcification inhibitor.
3. Gla domain but not proteolytic activity is required for VSMC calcification inhibition by PT.
4. PT accumulates at sites of calcification and PT circulation level is reduced in patients with vascular calcification.
5. VSMC exosomes display TF and PS-dependent thrombogenic activity.

Table 1. Bovine proteins in human VSMC exosomes detected by mass spectrometry.

VSMCs were incubated in DMEM supplemented with 0.1% BSA (CTRL, control) and 2.7mM Ca/2.5mM P (Ca/P) for 16h and exosomes isolated from cell media by differential centrifugation, *******proteins with Gla domain.*

Protein	Molecular Weight	Number of Assigned Spectra		Number of Unique Peptides		Percent Coverage	
		CTRL	Ca/P	CTRL	Ca/P	CTRL	Ca/P
Calcification inhibitors							
a-2-HS-glycoprotein	38	383	469	9	11	31	31
Transport Proteins							
Serum albumin	69	2834	2824	46	46	65	66
Matrix proteins							
Fibronectin	272	372	362	26	24	14	12
Coagulation proteins							
Prothrombin*	71	14	19	4	4	7.5	6.9
Factor X*	55	4	16	3	1	9.3	2.4
Factor V	249	10	12	4	3	1.9	1.5
Factor IX*	47	0	4	0	2	0	7.5
Protein C*	51	9	6	1	2	2.4	4.2
Protein S*	75	0	9	0	4	0	5.2

Table 2. Characterization of a patient cohort with chronic kidney disease (CKD), which were studied for their plasma concentrations of PT in relation to the presence of vascular calcification at the iliac/femoral arteries detected by plain X-ray of the pelvis.

	All	With vascular calcification	Without vascular calcification
Number	81	44	37
Gender (male/female)	40/41	25/19	15/22
Age (years)	55.38±9.33	56.43±9.46	54.14±9.15
BMI (kg/m^2)	23.47±3.40	23.32±3.03	23.66±3.82
Arterial hypertension	68 (84%)	35 (80%)	33 (89%)
Smoking	25 (31%)	17 (39%)	8 (22%)
Diabetes mellitus	7 (9%)	6 (14%)	1 (3%)
Years on dialysis	8.72±4.40	9.59±4.17	7.68±4.49
Cholesterol (mg/dl)	5.35±1.22	5.13±0.81	5.60±1.55
Triglycerides (mg/dl)	2.31±1.36	2.18±1.04	2.46±1.67
PT (%)	88.36±23.31	82.20±23.09	95.70±21.66*
			*p=0.0086 (Student's T-test); PT, prothrombin.