Vitamin K antagonist use induces calcification and atherosclerotic plaque progression resulting in increased hypercoagulability

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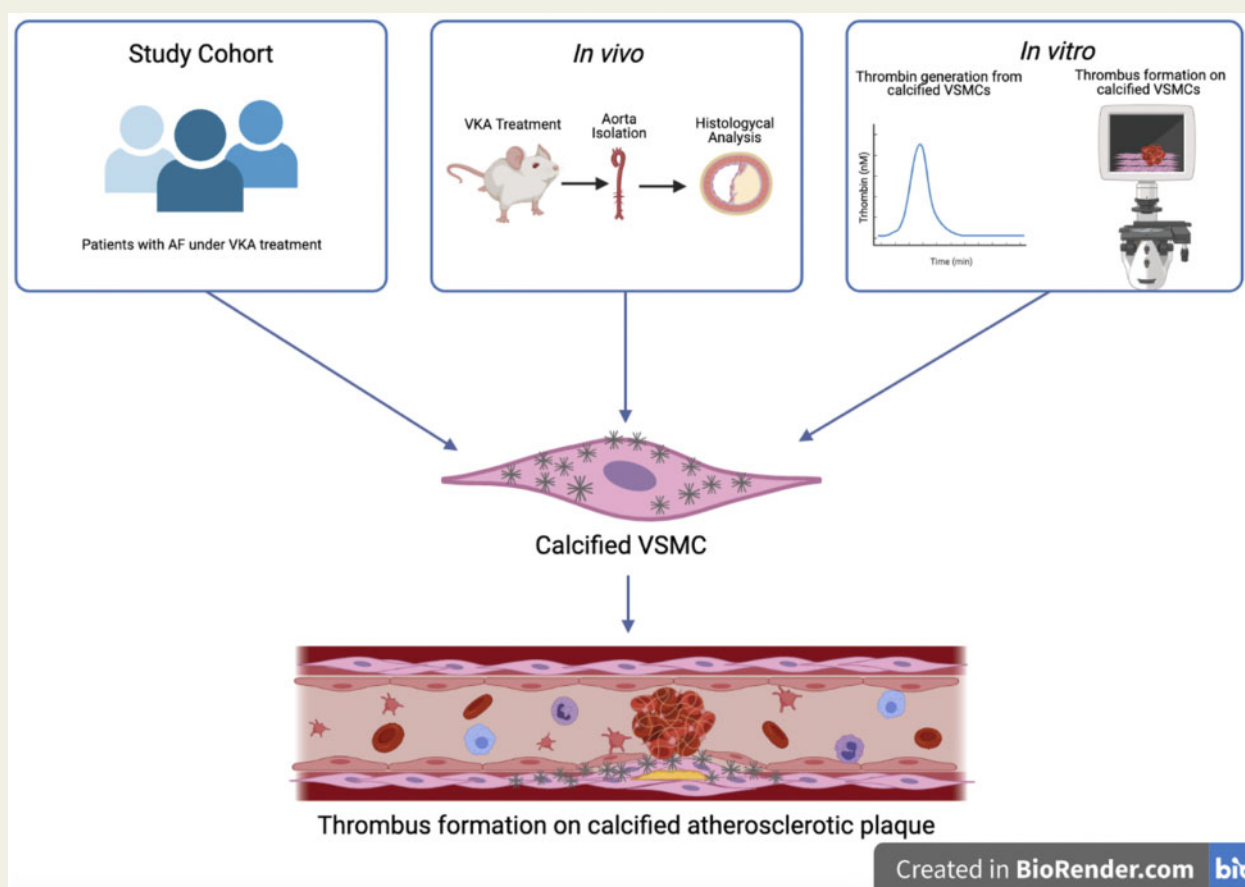|	
Aims	Vascular calcification is a hallmark of atherosclerotic burden and can predict the cardiovascular outcome. Vitamin K antagonists (VKA) are widely used anticoagulant drugs to treat patients at risk of arterial and venous thrombosis but are also associated with increase vascular calcification progression. We aim to unravel the paradox that VKA suppresses plasma coagulation but promotes vascular calcification and subsequent atherosclerosis-dependent coagulability of the vessel wall.
Methods and results	*Apoe*⁻/⁻ mice were placed on western-type diet enriched with the VKA warfarin for 18 weeks to measure atherosclerotic plaque burden, calcification, and coagulation. Patients (*n* = 54) displaying paroxysmal atrial fibrillation with a low cardiovascular risk, who were treated with VKA were included to measure pre-thrombotic state. Finally, primary vascular smooth muscle cells (VSMC) derived from human tissue explants were used for *in vitro* experiments. In *Apoe*⁻/⁻ mice, VKA increases both atherosclerotic plaque size and calcification. Higher plaque calcification was associated with increased plasma levels of thrombin-antithrombin and factor IXa-antithrombin complexes in mice and patients treated with VKA. Mechanistically, phenotypic switching of VSMC into synthetic VSMC promotes thrombin generation, which is enhanced in a tissue-factor (TF)-dependent manner by VSMC calcification. Moreover, calcified VSMC exposed to whole blood under flow significantly enhanced platelet deposition and TF-dependent fibrin formation.
Conclusions	Oral anticoagulation with VKA aggravates vascular calcification and atherosclerosis. VSMC phenotype differentiation impacts coagulation potential in a TF-dependent manner. VKA-induced vascular calcification increases hypercoagulability and could thereby potentially positively affect atherothrombosis.

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Graphical Abstract

Study Cohort — Patients with AF under VKA treatment

In vivo — VKA Treatment → Aorta Isolation → Histologycal Analysis

In vitro — Thrombin generation from calcified VSMCs; Thrombus formation on calcified VSMCs

Calcified VSMC

Thrombus formation on calcified atherosclerotic plaque

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Introduction

Vascular calcification is clinically assessed by computed tomography (CT), and is currently used as an independent predictor of cardiovascular burden.[1] Patients displaying vascular calcification are four times more likely to suffer from cardiovascular events.[2] Additionally, also an annual increase in vascular calcification has been found to be predictive for the risk of cardiovascular disease.[3]

The presence of micro-calcifications is strongly associated with plaque progression and vulnerability.[4] While micro-calcification is a key feature of high-risk atheroma plaques, the contribution of macro-calcification to plaque vulnerability is not clear.[5] The rationale for this comes from studies with intensive lipid-lowering therapy, showing a reduced risk of cardiovascular events and at the same time an accelerated progression of vascular calcification.[6] However, recently it has been put forward that also macro-calcification provides rupture stresses by stiffness mismatch.[7]

Vitamin K antagonist (VKA) treatment is used to reduce the risk of recurrent thrombosis but as a side effect accelerates vascular calcification.[8] Recently, VKA treatment was shown to be associated with increased arterial stiffness and decreased cardiac systolic function.[9] Experiments in rodents have shown that VKA treatment shifts atherosclerotic plaques towards a vulnerable phenotype.[10] On the other hand, accelerated thrombin formation is predictive for the presence and severity of atherosclerosis.[11]

Atherosclerosis is multifactorial, characterized by inflammation and calcification.[1,12] Activated coagulation factors are recognized as pro-inflammatory, pro-atherogenic, and plaque destabilizing.[13] Early atherosclerotic plaques exhibit an enhanced pro-coagulant state[14] and activated coagulation factors can activate cellular protease-activated receptors, thereby inducing inflammation, apoptosis, calcification, and fibrosis.[15] This indicates a strong dependency and connection between coagulation and atherosclerosis, termed athero-thrombosis. Atherothrombosis is characterized by plaque rupture or

progressive stenotic narrowing of the lumen to such an extent that platelet thrombi can occlude the vessel lumen completely. We aimed in this study to unravel the apparent paradox that VKA lowers coagulation tendency and induces atherosclerotic plaque progression and calcification, yet increasing hypercoagulability.

Results

$Apoe^{-/-}$ mice were fed for 18 weeks a western-type diet (WTD; control), or WTD supplemented with warfarin, in order to determine the effect of warfarin on plaque progression and calcification (Supplementary material online, Figure S1A). After 18 weeks, plasma triglycerides and cholesterol as well as animal body weights were similar between the two groups (Supplementary material online, Table S1). Warfarin-treated mice showed high plasma levels of warfarin, in contrast to the control mice (Supplementary material online, Table S1), thus confirming adequate uptake of warfarin from the diet.

After 18 weeks of diet, the atherosclerotic plaques from mice treated with warfarin showed significantly increased vascular calcification, as compared to the control group (Figure 1A). Additionally, the

warfarin diet for 18 weeks caused an enlarged atherosclerotic plaque size, compared to the control diet ($P < 0.05$, Figure 1B).

Warfarin is known to interfering with the recycling of vitamin K resulting in the accumulation of uncarboxylated proteins. To verify this, we quantified uncarboxylated matrix Gla protein (ucMGP) in aortic atherosclerotic plaques.[10] The ucMGP expression was significantly higher in the warfarin group (Supplementary material online, Figure S1E). In addition, levels of ucMGP strongly correlated with extent of plaque calcification ($r = 0.82$, $P < 0.01$; Supplementary material online, Figure S1F).

Using cultured VSMC, we tested whether warfarin addition also could affect MGP carboxylation in vitro. Indeed, in cultured VSMC warfarin caused a time-dependent increase in ucMGP at the expense of carboxylated MGP (cMGP) (Supplementary material online, Figure S1C). Additionally, warfarin increased the calcification of VSMC in a dose-dependent way (Supplementary material online, Figure S1D).

In patients with coronary artery disease, the severity of atherosclerosis, as measured by computed tomographic angiography, has been linked to accelerated thrombin generation.[11] To evaluate this in the present setting, we determined the levels of thrombin-antithrombin (TAT) and factor IXa: antithrombin (FIXa:AT)
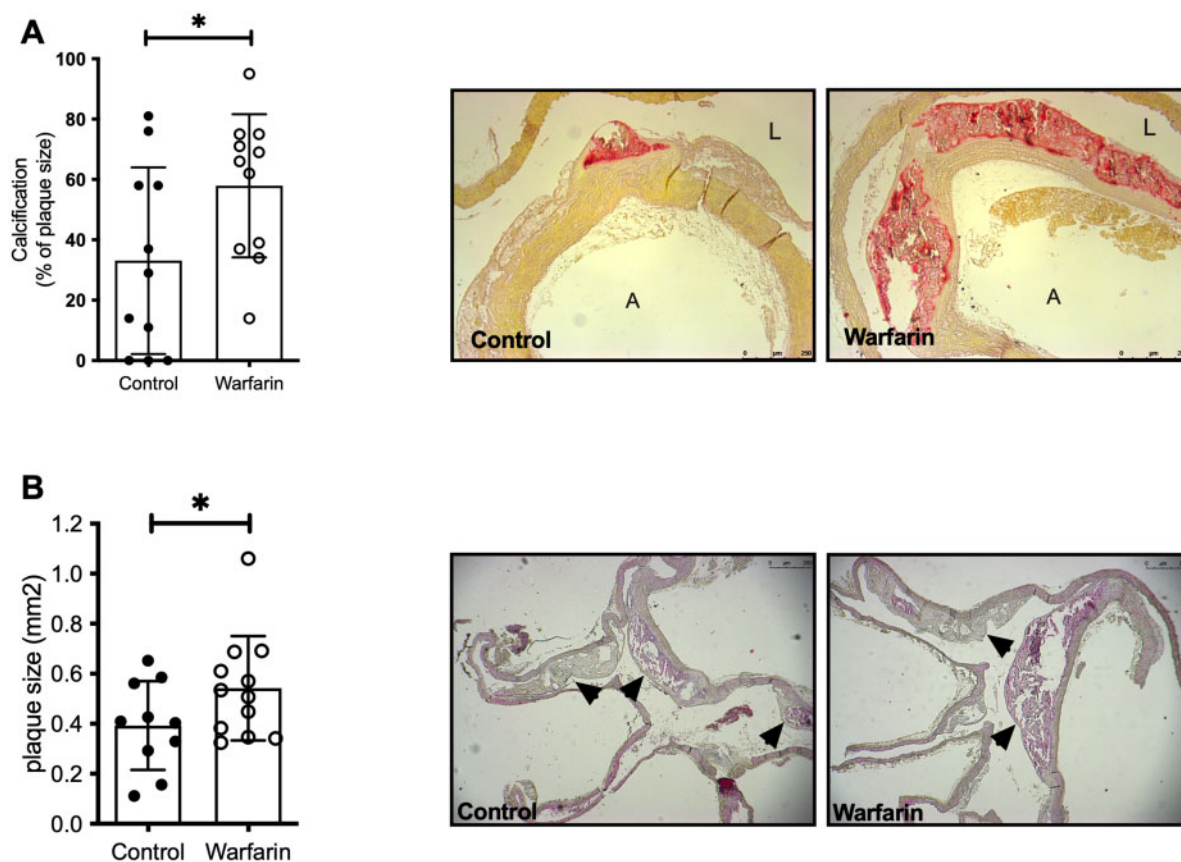

Figure 1 Warfarin treatment increases plaque calcification. (A) 18 weeks of treatment demonstrated that warfarin increased calcification compared to control treatment detected by Alizarin Red S staining. Scale bar = 250 µm. Control (n = 11) and warfarin (n = 11) (longitudinal arch sections; A, Adventitia; L, Lumen). (B) Plaque size was significant increased after 18 weeks of warfarin treatment compared to control treatment. Scale bar = 500 µm. Control (n = 10) and warfarin (n = 12) (black arrows indicate atherosclerotic plaque areas). Number of analysis varied from 10 to 12 due to technical errors and missing of data. Data were analysed using Mann–Whitney U test.

complexes in plasma from control- and warfarin-treated animals. Elevation in these complexes points to the presence of a pre-thrombotic state.[16] Interestingly, levels of TAT but not of FIXa:AT complexes were markedly increased in mice treated with warfarin (*Figure 2A,B*). To assess a possible relation to extent of plaque calcification, we stratified control and warfarin-treated mice based on median plaque calcification percentage (median; 58% calcification of plaques). We noted a significant increase in FIXa:AT levels (p < 0.01) and a close to significant increase in TAT levels (*P* = 0.068) in animals with calcification exceeding the median (*Figure 2C,D*).

Phenotypic switching of VSMC is known to play a crucial role in maintaining the structural integrity of the blood vessel,[17] and in vascular calcification.[18] We used lysates of contractile, synthetic or calcified VSMC to investigate the consequences of VSMC phenotypic switching on coagulation stimulation in human pooled normal plasma, by measuring thrombin generation parameters using the CAT assay. Typically, synthetic VSMC showed a shorter lag-time and increased thrombin peak height, velocity index, and endogen thrombin potential (ETP) when compared to contractile VSMC (*Figure 3A–E*). Lysates from calcified VSMC showed a slightly shorter lag-time and higher peak height and velocity index when compared to synthetic VSMC (*Figure 3F–J*). In order to eliminate the role of contact activation (intrinsic pathway), further thrombin generation experiments were performed with factor XII deficient plasma. In the absence of

Figure 2 Vascular calcification induces a pre-thrombotic state. (*A*) Circulating FIXa:AT levels remained similar between warfarin and control treated mice after 18 weeks. Control (*n* = 20) and warfarin (*n* = 22). (*B*) Warfarin treated mice had significantly increased circulating TAT levels as compared to control mice. Control (*n* = 19) and warfarin (*n* = 22). (*C*) Stratification of all mice by calcification revealed a significant increase in circulating FIXa:AT ratio levels in mice with higher than the median calcification score. Median < (*n* = 18) and median > (*n* = 21). (*D*) Stratification results in a borderline significant increase (*P* = 0.068) of circulating TAT levels in mice with higher than median calcification score. Median < (*n* = 17) and median > (*n* = 21). Data were analysed using Mann–Whitney *U* test.

factor XII (*Figure 4*), thrombin generation parameters were comparable to those obtained in pooled normal plasma (*Figure 3*). However, administration of ASIS, blocking the tissue factor (TF)-dependent coagulation pathway, prolonged the lag-time and reduced the thrombin peak height, ETP, and velocity index in all VSMC phenotypes (*Figure 4A–J*). These findings were confirmed for synthetic and calcified VSMC using factor VII deficient plasma in the presence or absence of the factor XIIa inhibitor, CTI, or ASIS (Supplementary material online, *Figure S2*). Here, the use of factor VII deficient plasma or the addition of ASIS reduced the thrombin peak height and prolonged the lag time. Since the extrinsic pathway seems key in VSMC-mediated activation of thrombin we measured TF procoagulant activity of VSMC lysates. TF activity was significantly increased in synthetic VSMC compared to contractile VSMC (*Figure 4K*). Moreover, calcified synthetic VSMC showed a further increase in TF activity, when compared to non-calcified synthetic VSMC (*P* < 0.05; *Figure 4K*).

To further prove the TF-mediated pro-coagulant activity, we investigated the coagulation ability of calcified or non-calcified synthetic VSMC when subjected to whole blood flow. Markedly, calcified VSMC showed an increased thrombin-dependent fibrin formation and denser platelet thrombus formation (*Figure 5A,B*). Interestingly, a short-term treatment of synthetic VSMC with warfarin in the presence of calcium was sufficient to significantly enhance fibrin and thrombus formation (*Figure 5D,E*) suggesting that initiating calcification of VSMC promotes coagulation. To investigate whether the fibrin formation was induced via the intrinsic or extrinsic coagulation pathway, CTI or ASIS was added to the blood. Using calcified VSMC, CTI treatment resulted in an attenuation of fibrin formation (*P* < 0.05), whereas ASIS pre-treatment completely prevented fibrin formation (*P* < 0.01). Moreover, ASIS significantly reduced platelet thrombus formation (*Figure 5C*). Together, these data indicate that VSMC calcification enhances the cellular coagulant activity, predominantly in a TF-dependent manner.

To further evaluate if patients with the presence of vascular calcification show evidence for a pre-thrombotic state, we analysed the TAT and FIXa:AT levels in a subset of a previously published cohort of VKA treated patients with or without CAC.[19] It appeared that the presence of CAC (Agatston score > 0) is significantly associated with higher FIXa:AT levels, but not with TAT levels (*Figure 6A,B*, respectively). Dividing patients with CAC in low Agatston score (1–50) and high Agatsan score (150–647) did not show any association with FIXa:AT or TAT levels (*Figure 6C,D*, respectively). Future studies should evaluate the CAC progression in relation to the duration of VKA treatment and the pre-thrombotic state.

Discussion

In the present study, we demonstrate that VKA treatment increased both progression and calcification of atherosclerotic plaques in atherosclerosis prone mice. Increased calcification was accompanied by overall increased plasma levels of TAT and FIXa:AT. We show that calcified synthetic VSMC expresses higher levels of TF-activity as compared to synthetic and contractile VSMCs. Furthermore, calcified VSMC caused significantly more platelet aggregation and fibrin formation when exposed to whole blood under flow as compared to non-
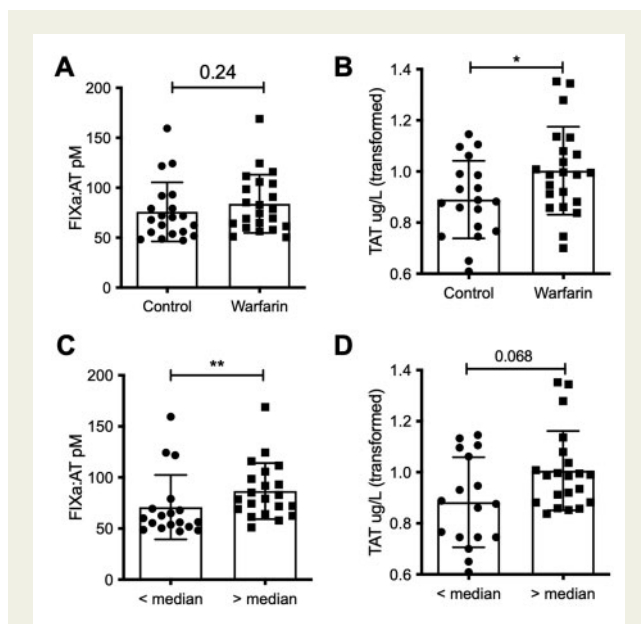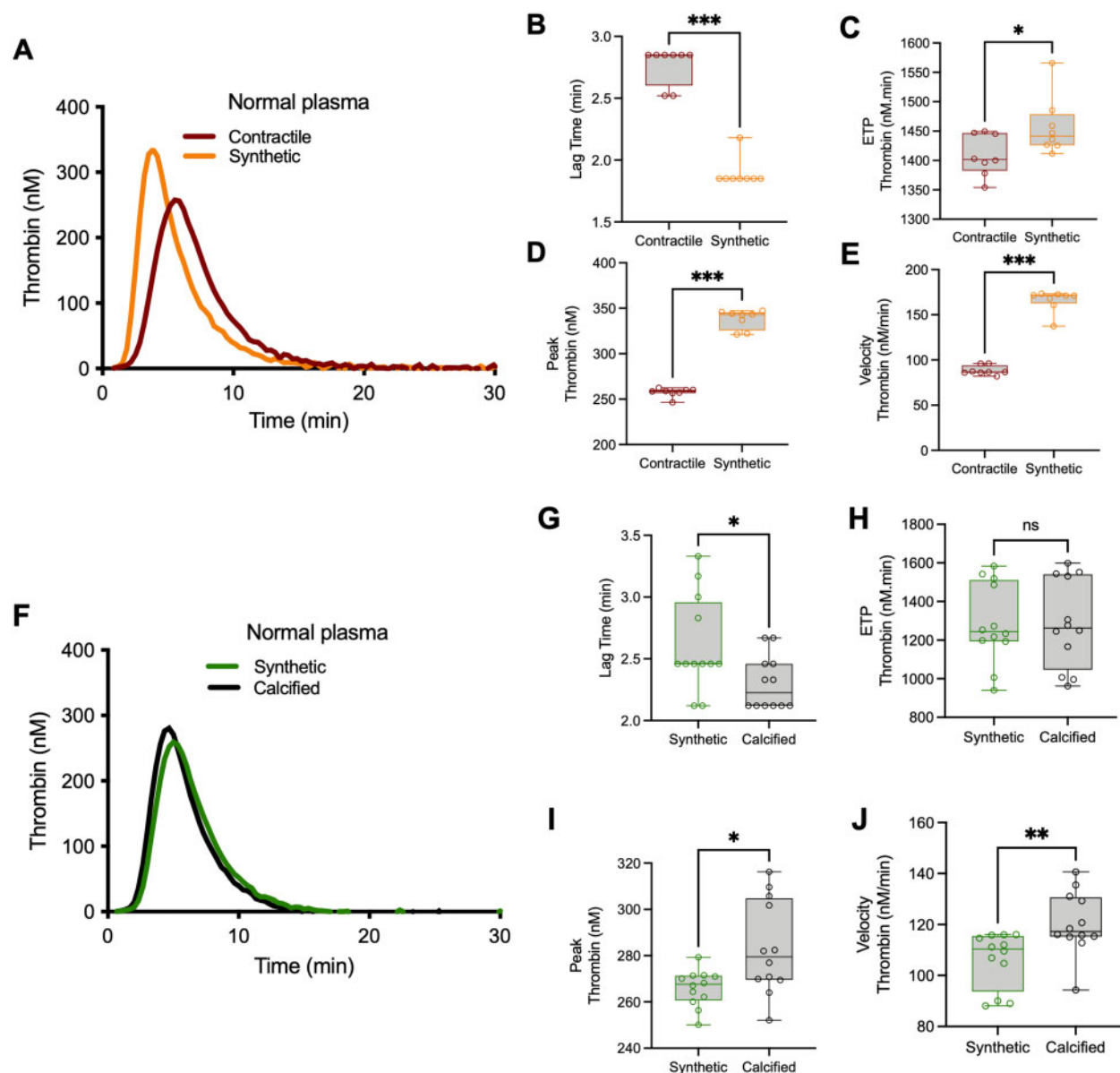
Figure 3 Thrombogenicity of vascular smooth muscle cell phenotype in normal plasma. (*A*) Using human normal pooled plasma, both synthetic (orange) and contractile (red) VSMC were inducers of thrombin generation. Synthetic VSMC showed a shorter lag time ($n = 8$) (*B*), slightly increased endogen thrombin potential (ETP) ($n = 8$) (*C*) and higher peak height ($n = 8$) (*D*) and velocity index ($n = 8$) (*E*) compared to contractile VSMC. (*F*) Calcified VSMC (black) demonstrated a shorter lag time ($n = 12$) (*G*), similar ETP ($n = 12$) (*H*), higher peak height ($n = 12$) (*I*) and velocity index ($n = 12$) (*J*) as compared to synthetic VSMC (green). All experiments were done with at least three biological replicates, with quadriplo technical replicates. Data were analysed using Mann–Whitney *U* test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

calcified VSMC. Taken together our data indicate that calcification renders atherosclerotic lesions more prone to cause atherothrombosis.

CAC strongly associates with atherosclerosis and the progression of CAC is a strong predictor of future adverse cardiac events.[3] VKA has been shown to increase vascular calcification.[11,20] Here we confirm that VKA treatment enhances atherosclerotic plaque calcification and size.[10] We noticed that VKA

treatment in mice resulted in smaller, yet more inflammatory atherosclerotic plaques in the early phase of atherogenesis (Supplementary material online, *Figure S1B*). We have previously shown that micro-calcification can precede atherosclerosis.[4] Although we did not detect calcification at the early time point, it has been shown that inactive uncarboxylated MGP strongly correlates with micro-calcification and that these plaques are more pro-inflammatory.[4]
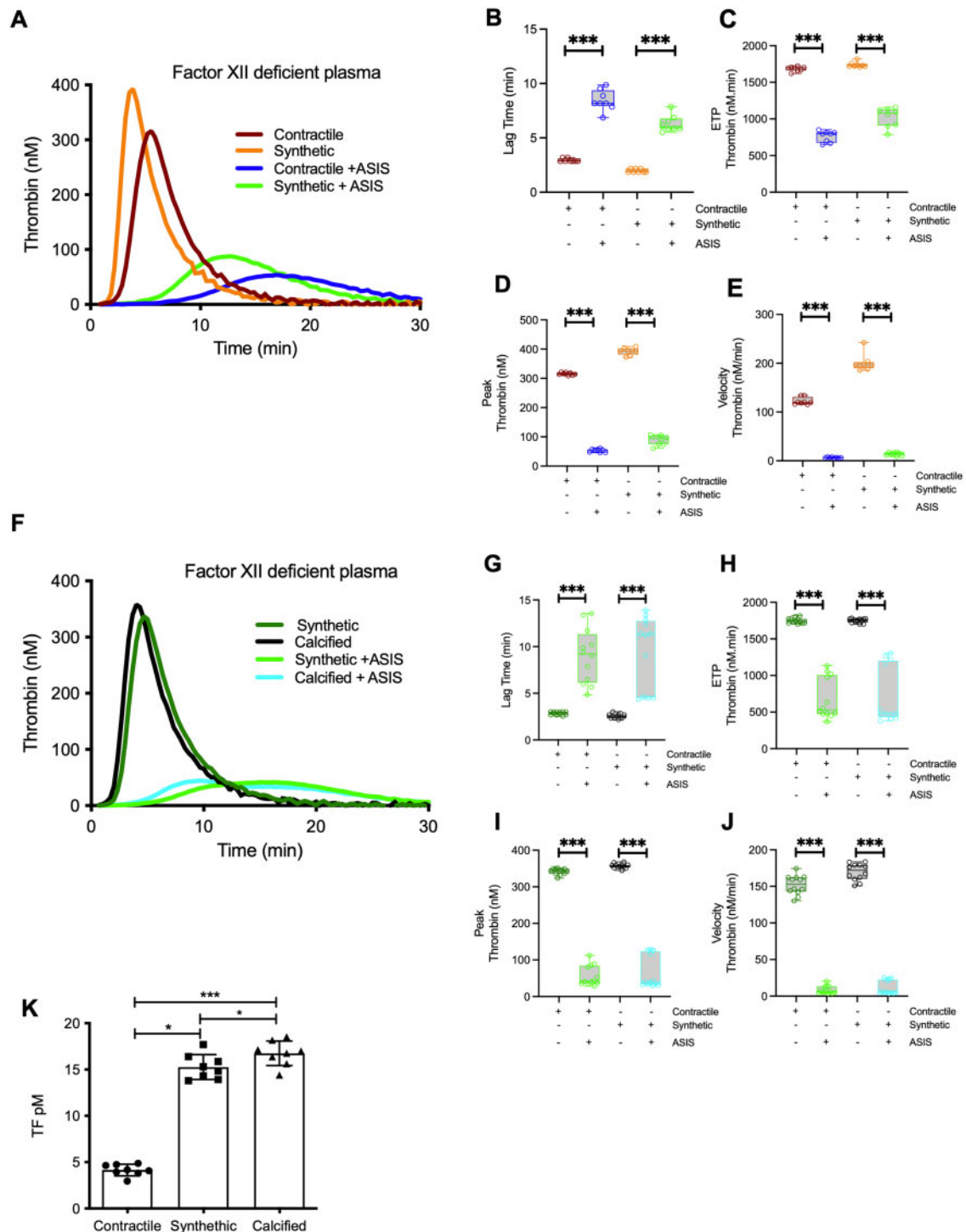
Figure 4 Thrombogenicity of vascular smooth muscle cell phenotype in Factor XII deficient plasma. The contribution of the phenotype of vascular smooth muscle cells to intrinsic and extrinisic activation of coagulation was assessed in factor XII deficient plasme. Thrombin generations of contract-ile, synthetic and calcified VSMC in factor XII deficient plasma were comparable to those obtained in normal pooled plasma (*A and F*, and *Figure 3*). Inhibition of the extrinsic pathway through addition of activated site inhibitor seven (ASIS) in factor XII deficient plasma prolonged the lag time (*n* = 8; B and *n* = 12; *G*) and reduced endogen thrombin potential (ETP) (*n* = 8; *C and n* = 12; *H*), peak height (*n* = 8; *D and n* = 12; *I*) and velocity index (*n* = 8; *E and n* = 12; *J*) of all VSMC phenotypes synthetic. (*K*) Contractile VSMC showed significant lower pro-coagulant TF activity levels as compared to synthetic and calcified VSMC. Calcified VSMC show a significant increase in pro-coagulant TF activity as compared to synthetic VSMC. Control (*n* = 8), synthetic (*n* = 8), calcified (*n* = 8). All experiments were done with at least three biological replicates, with quadriplo technical replicates. Data were analysed using Mann–Whitney *U* test (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).
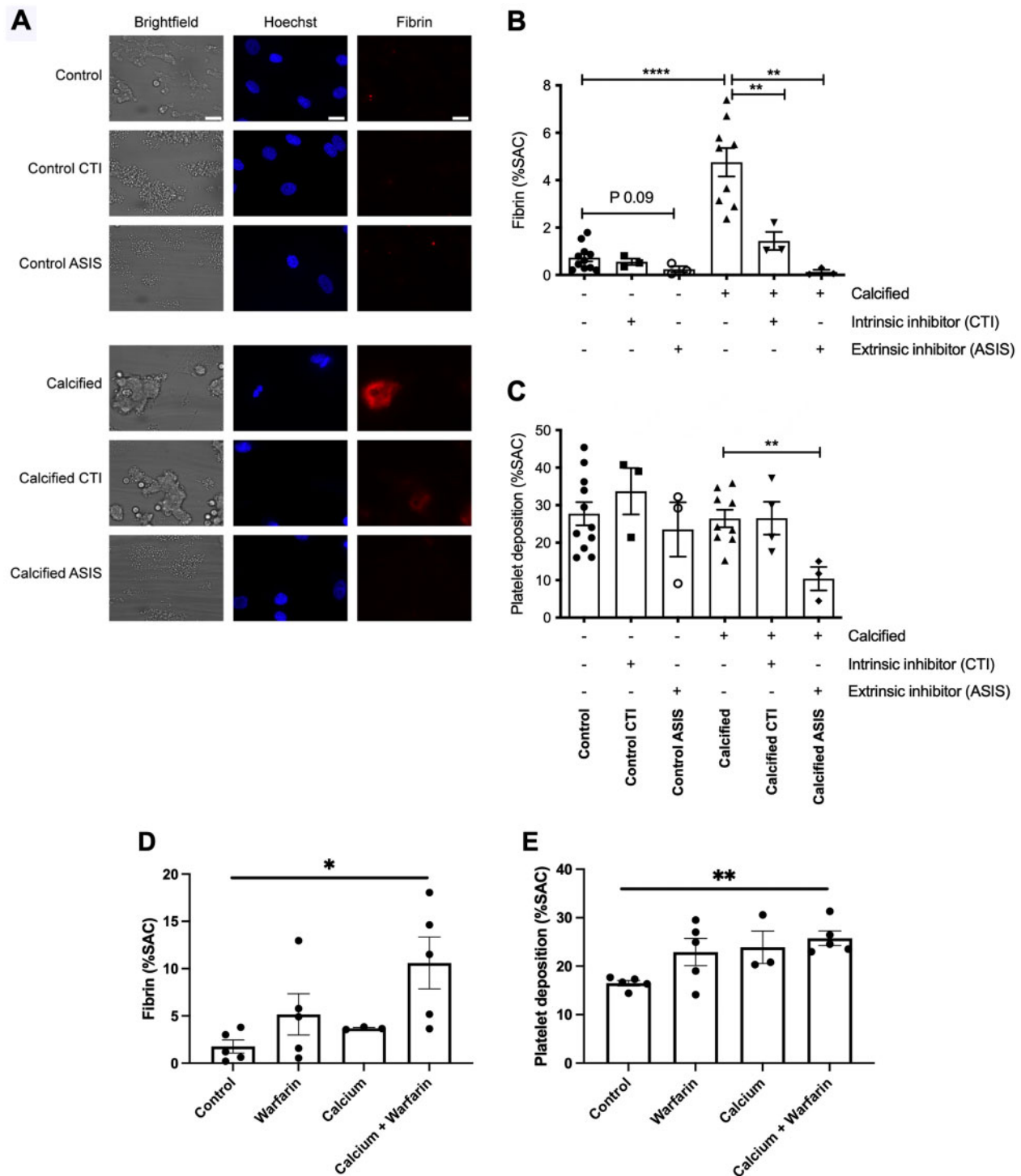
Figure 5 Thrombogenicity of vascular smooth muscle cells under flow conditions. (*A*) Representative images of platelet thrombus formation (brightfield), VSMC nuclei (Hoechst33342, blue) and AF647-fibrin formation (red) on calcified synthetic VSMC under blood flow. (*B*) Quantification of fluorescent fibrin formation indicates an increase in the presence of calcification, and a reduction in the presence of either CTI or ASIS. (*C*) Platelet deposition on calcified VSMC was reduced in the presence of ASIS. Scale bar = 20 μm. (*D*) Quantification of fluorescent fibrin indicates an increase of fibrin generation in VSMCs treated with warfarin and calcium (*P* = 0.0337). (*B*) Quantification of platelet deposition of synthetic VSMCs under flow. Cells treated with warfarin and calcium show an increase in platelet deposition (*P* = 0.0059). All experiments were done with at least three biological replicates, with duplicate technical replicates. Data were analysed using Mann–Whitney *U* test and Dunnett's multiple comparison test.
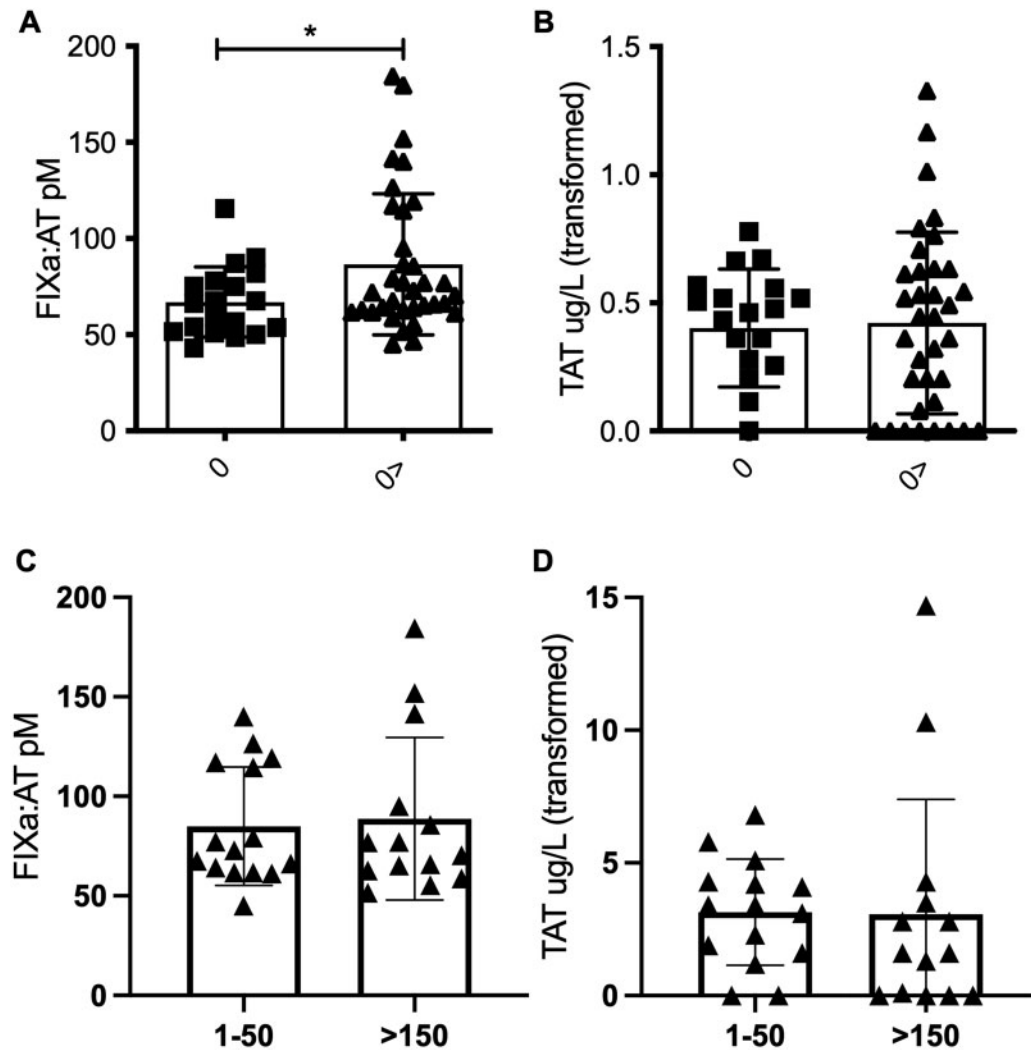
Figure 6 Vascular calcification promotes a pre-thrombotic state in patients on vitamin K-antagonist (VKA). Patient data include 54 patients on VKA (acenocoumerol) treatment with a mean duration of 44 ± 26 month. VKA treated patients with coronary calcification (Agatston score > 0; *n* = 35) compared to no coronary calcification (Agatston score = 0; *n* = 19) showed no difference on circulating TAT levels (*B*), but significantly increased circulating FIXa:AT levels (*A*). Dividing the Agatston scores >0 in low (Agatston score 1–50) and high (150–647) did not show any significance for TAT levels (*D*) or FIXa:AT (*C*). Data were analysed using Mann–Whitney *U* test.

Recently it has been shown that VKA treatment enhances calcification and increases plaque volume in patients with atrial fibrillation undergoing coronary CT angiography with 1-year follow-up as compared to apixaban.[21] Statin treatment, however is associated with a favourable cardiovascular outcome and increased vascular calcification, yet a lower plaque volume.[22] Our data demonstrate that VKA treatment increases both calcification and atherosclerotic plaque volume, indicating different mechanisms and impacts on plaque stability.[23]

In the healthy vessel wall, VSMC exhibit a contractile phenotype to support vascular tone. Upon injury, VSMC switch phenotype to facilitate vascular remodelling.[24] Synthetic VSMC are key in vascular calcification.[25] VKA-induced calcification is most likely mediated via impaired carboxylation of MGP as well as prothrombin.[26] We show that VSMC in vitro produce inactive ucMGP in response to VKA treatment, subsequently accelerating calcification. *In vivo*, ucMGP positivity in the atherosclerotic plaque significantly correlated with the amount of atherosclerotic plaque calcification. It is noteworthy to mention that we visualized atherosclerotic calcification using Alizarin Red S, a calcification stain that strongly correlates with the PET tracer NaF[18] that is known to detect active calcification.[27]

Patients with no or very low CAC scores have a lower risk on future clinical CVD events compared to patients with higher CAC scores.[28] Moreover, the CAC score is used as a marker for atherosclerotic plaque burden.[1] This confront us with the intriguing and crucial question as to whether calcium deposits in atherosclerotic
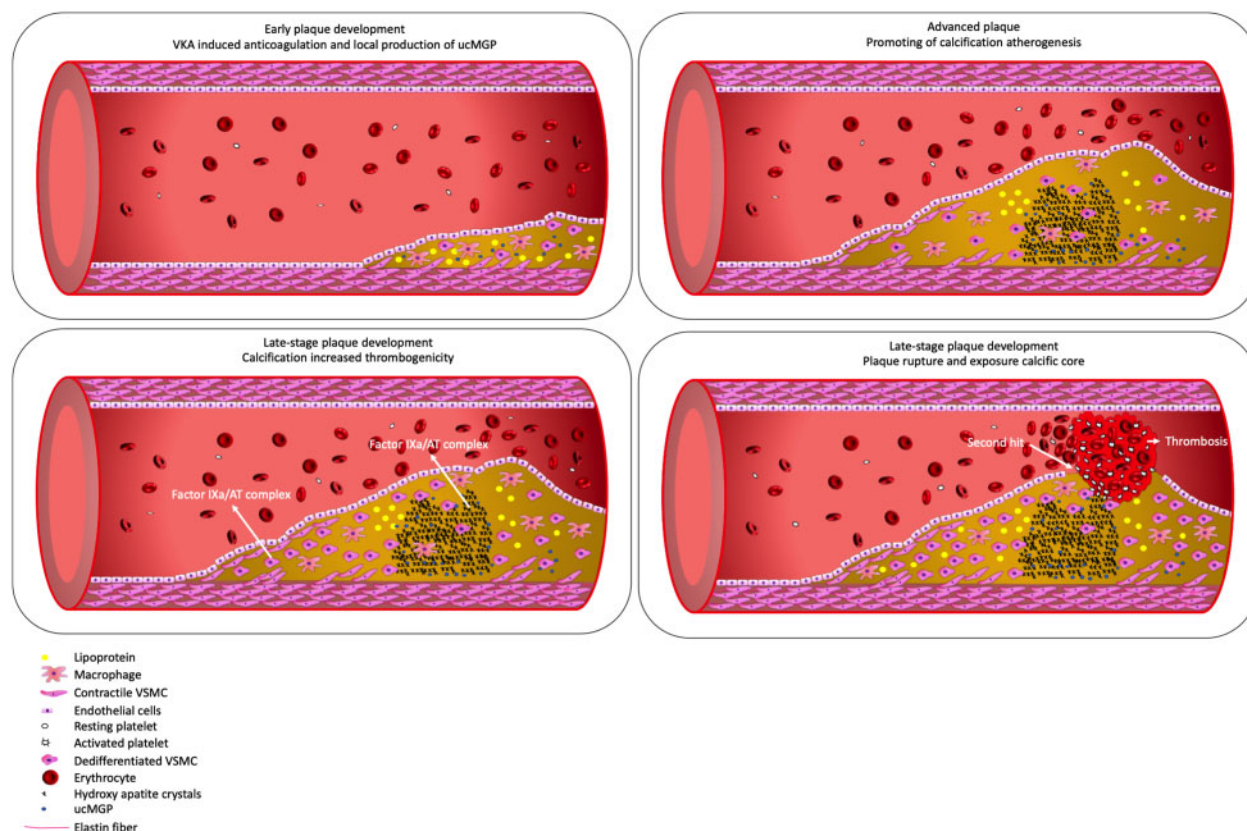
Figure 7 Short-term treatment of VKA induces systemic and local vitamin K deficiency. Long-term VKA treatment aggravates atherogenesis and results in increased vascular calcification. Vascular calcification induces a pre-thrombotic state priming for an increased risk on atherothrombosis. (*A*) (1) VKA induce local vitamin K deficiency. (2) Vitamin K deficiency initiates micro-calcification in the vessel wall. (3) Vascular calcification promotes vessel stiffening causing local wall damage and stress. (4) Plaque development is initiated by local inflammation. (*B*) (1) Calcification causes vessel wall stiffening. (2) VSMCs proliferate and are activated thereby expressing tissue factor. (3) VKA treatment causes further plaque progression and calcification. (4) Local mechanical changes occur which promotes flow perturbations. (*C*) (1) Calcified plaques favour a pre-thrombotic coagulation state due to the formation of FIXa:AT and TAT complexes. (2) This pre-thrombotic state feeds back into the vessel thereby promoting further plaque progression resulting in (3) VSMC proliferation and (4) calcification of the vasculature. (*D*) (1) Upon plaque erosion, calcified material and activated VSMCs are exposed to the blood, causing platelet activation and fibrin deposition leading to (2) thrombus formation.

plaques contribute to plaque stability or increase plaque instability. Upon rupture of an atherosclerotic plaque, thrombosis occurs which is termed atherothrombosis, causing myocardial infarction and stroke, the most common causes of death in the Western world.[29]

Because trace amounts of thrombin are continuously formed under physiological conditions, complexes of active serine proteases with their natural inhibitor, antithrombin, are always detectable. FIXa:AT reflects an early part of the coagulation cascade, immediately prior to factor X and prothrombin conversion. Additionally, TAT complex is a marker of downstream coagulation activity. Patients with increased CAC were shown to have increased FXIIa levels[30] and TAT complex levels.[11,31] In low-risk paroxysmal atrial fibrillation patients increased FIXa:AT suggests a pre-activation state with sufficient down-stream inhibition of coagulation and indicative for hypercoagulability.[16] Subsequently, hypercoagulability promotes atherogenesis and atrial fibrillation.[32] We show that FIXa:AT ratio

was significantly increased in presence of increased vascular calcification. In contrast to previous reported elevated TAT complexes for patients with increased CAC,[30] this association was not seen in the current study. One explanation could be the limited number of subjects included in this study compared to the larger population and more distinct CAC classification in the previous study. Nevertheless, increased FIXa:AT complexes suggest a pre-activation state of coagulation in association with increased CAC, without obvious downstream activation of prothrombin. Overall, our data indicate that the presence of calcification promotes a pre-thrombotic state.

The thrombotic complications in atherosclerosis can be due to plaque rupture and plaque erosion. Rupture of the vulnerable plaque exposes blood with its coagulation factors to thrombogenic material residing within the plaque. However, improved control of traditional risk factors such as cholesterol and inflammation has reduced the incidence of disruptive events leading to thrombus formation.[33]

However, vascular calcification cannot be treated yet. Additionally, plaque erosion, that is the loss of the endothelial coverage on atherosclerotic plaques, might also have a great clinical impact, by exposure of the underlying plaque material to the blood, including calcification. In peripheral arterial vessels, the presence of vascular calcification leading to stiffening of the vasculature strongly correlates with thrombotic occlusions.[34] Upon endothelial disruption, TF is released which ignites the extrinsic coagulation pathway via activation of FVII.[35] VSMC normally express low levels of TF but during atherogenesis, TF is expressed abundantly.[36] In the atherosclerotic plaque, VSMC acquire a synthetic phenotype. To unravel the mechanism by which calcification activates coagulation, we used VSMC *in vitro*. Here we show that synthetic VSMC harbour significantly more TF activity compared to contractile VSMC and that calcified VSMC have even more TF activity, which is in line with increased ETP.

Platelets play a pivotal role in vascular occlusive disease where they contribute to fibrin formation and inflammation.[37] It has been shown that membrane-associated platelet polyphosphate is condensed into insoluble spherical nanoparticles with divalent metal ions such as calcium and that these calcium-phosphate particles activate FXII.[38] We showed that the initiation of coagulation was in part mediated via the intrinsic pathway but completely abolished by inhibition of the extrinsic pathway. The importance of TF activity of VSMC was shown in mice lacking VSMC derived TF, which had reduced thrombus formation after ferric chloride injury.[39] It has also been shown that VSMC exposed to flow reveal a burst of TF expression,[40] confirming involvement of the extrinsic coagulation pathway. Moreover, in aortic valves, TF expression was increased in calcified compared to non-calcified regions,[41] suggesting that calcification correlates with TF expression. Local calcified vascular areas might initiate fibrin formation, which is known to promote atherogenesis and plaque instability.[42] These data were confirmed by our in vitro flow experiments, showing that calcified VSMC caused significantly more fibrin formation. Taken together, this suggests that atherosclerotic calcified plaques harbour more TF activity, thereby driving atherothrombosis upon plaque rupture.

A limitation of our study is the limited number of low-risk AF patients included who were treated with VKA and of which CAC quantification was available. The patients with low-risk AF included in our study had only modest increased CAC levels. The low number of patients only allowed us to test the hypothesis of increased coagulation in patients with calcification, without drawing any hard conclusion. Larger clinical trials, including patients treated with non-vitamin K antagonist oral anticoagulants (NOACs), should confirm our findings.

In conclusion, our results show that long-term VKA treatment impacts VSMC thereby aggravating calcification and atherosclerotic plaque progression. Within the atherosclerotic plaque, VSMC switch phenotype and subsequently promote calcification and TF-dependent coagulation. Calcification of atherosclerotic plaques might be a reaction to inflammation, but it also renders the lesion more prone to atherothrombosis following rupture or exposure to the blood. Further, calcified vasculature exposed to blood significantly increased platelet thrombus formation and fibrin deposition (*Figure 7*). Our findings provide additional support to the observed association between CAC and risk for future cardiovascular events.

Lead author biography

Rick studied Biomedical Sciences at Maastricht University and received his MSc degree in 2014. After graduation, interest in the vascular remodelling and nutritional intervention drove Rick to start an Industrial PhD at the department of Biochemistry, Maastricht University in collaboration with Nattopharma ASA. In the lab of Prof. Leon Schurgers, Rick researched the role of vascular smooth muscle-cell phenotypic switching in vascular calcification. After obtaining his PhD degree, Rick continued his career in the industry where he contributes to development of pharmaceuticals in the field of immunity.

Supplementary material

Supplementary material is available at *European Heart Journal Open* online.

Funding

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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