Off-target effects of oral anticoagulants – vascular effects of vitamin K antagonist and non-vitamin K antagonist oral anticoagulant dabigatran etexilate

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Abstract

Introduction: Vitamin K antagonists (VKA) and non-vitamin K oral antagonist anticoagulants (NOAC) are used in the clinic to reduce risk of thrombosis. However, they also exhibit vascular off-target effects. The aim of this study is to compare VKA and NOAC on atherosclerosis progression and calcification in an experimental setup.

Material and methods: Female Apoe−/− mice (age 12 weeks) were fed Western-type diet as control or supplemented with dabigatran etexilate or warfarin for 6 or 18 weeks. Vascular calcification was measured in whole aortic arches using µCT and [18F]-NaF. Atherosclerotic burden was assessed by (immuno)histochemistry. Additionally, in vitro effects of warfarin, thrombin, and dabigatran on primary vascular smooth muscle cells (VSMC) were assessed.

Results: Short-term treatment with warfarin promoted formation of atherosclerotic lesions with a pro-inflammatory phenotype, and more rapid plaque progression compared with control and dabigatran. In contrast, dabigatran significantly reduced plaque progression compared with control. Long-term warfarin treatment significantly increased both presence and activity of plaque calcification compared with control and dabigatran. Calcification induced by warfarin treatment was accompanied by increased presence of uncarboxylated matrix Gla protein. In vitro, both warfarin and thrombin significantly increased VSMC oxidative stress and extracellular vesicle release, which was prevented by dabigatran.

Conclusion: Warfarin aggravates atherosclerotic disease activity, increasing plaque inflammation, active calcification, and plaque progression. Dabigatran lacks undesired vascular side effects and reveals beneficial effects on atherosclerosis progression and calcification. The choice of anticoagulation impacts atherosclerotic disease by...
The NOAC dabigatran etexilate (DE) was one of the first non-vitamin K oral antagonists on the market. DE is an oral prodrug that is rapidly converted by esterases to dabigatran, which is a direct, competitive inhibitor of thrombin (FIIa). Thrombin is a key enzyme in the coagulation cascade, ensuring the conversion of fibrinogen into fibrin. Thrombin has also a wide spectrum of effects directly on the vessel wall, which contribute to atherogenesis. Increased thrombin formation has been shown to aggravate atherogenesis. Inhibition of thrombin by dabigatran reduced plaque size and atherogenesis. Because dabigatran exhibits these beneficial effects on reducing plaque size, we addressed the question whether dabigatran also has an effect on plaque calcification. Using a preclinical experimental animal model of atherosclerosis, we assessed the short- and the long-term effects of the VKA warfarin and the NOAC dabigatran on atherosclerotic plaque calcification and atherogenesis.

2 | METHODS

2.1 | Experimental animals

All animal studies were performed under an approved protocol by the Ethics Committee for animal experiments of Maastricht University. Twelve-week-old female C57/BL6 Apoe−/− mice were purchased from Maastricht University and housed in climate-controlled spaces under 12-hour day/night cycle with ad libitum access to food and water. In our model, we used female mice because they develop more advanced atherosclerotic lesions. Mice were fed an irradiated (0.9 Mrad) vitamin K–deficient Western type diet (WTD: 0.25% cholesterol and 15% cocoa butter, derived from Altromin, Lage, Germany). WTD was supplemented with corn oil–dissolved vitamin K1 (5 mg/kg, Merck KGaA, Darmstadt, Germany) and was used as control diet or supplemented with DE, 7.5 mg/g (~22.5 mg/mouse/day), Boehringer Ingelheim, Biberach, Germany) for NOAC treatment. Additionally, WTD was supplemented with warfarin (3 mg/g warfarin (~9 mg/mouse/day); Merck KGaA, Darmstadt, Germany) and vitamin K1 (1.5 mg/g K1, Merck KGaA)
for VKA treatment. Vitamin K1, which counteracts warfarin’s effects predominantly in the liver and less in extrahepatic tissue, was additionally added to the warfarin diet to prevent bleeding.\textsuperscript{9,25} Animals were scored every day for well-being and bleedings because of the potential bleeding effects of warfarin and dabigatran.

Mice were randomly divided to receive WTD (control), DE, or warfarin (warfarin) supplemented food for 6 or 18 weeks (\(n = 12\) per treatment per time point; Figure 1A). Thereafter, mice were sacrificed, and blood was collected in 105 mM trisodium citrate via the portal vein. Plasma was prepared and frozen in aliquots at \(-80^\circ\text{C}\) until analysis. Next, the vasculature was washed with a sterile binding buffer (40 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), and 2.5 mM CaCl\(_2\), pH 7.3) via the left ventricle. Aortas were harvested, dissected, and fixed in 1\% (v/v) HEPES-buffered formaldehyde containing 150 mM saline, overnight at 4\(^\circ\text{C}\), before they were embedded in paraffin. Before sections were cut, paraffin embedded aortas were analyzed \textit{ex vivo} for calcification using \(\mu\)CT.

2.2 | Blood analysis

Warfarin and dabigatran plasma levels were determined using high-performance liquid chromatography and Hemoclot thrombin inhibitor assay (Hyphen Biomed, Neuville-sur-Oise, France), respectively. The prothrombin time of plasma, a measure for clotting tendency, was determined according to standard laboratory methods using the appropriate tests. Total plasma cholesterol and triglyceride levels were assessed with the Synchron LX20 (Beckman Coulter, Brea, CA) according to the manufacturer’s instructions.

2.3 | \([^{18}\text{F}]\text{-NaF}\)

Mice (\(n = 7, 6, \) and 8 for control, warfarin, and DE, respectively) were fasted for 2 hours before intravenous injection with \([^{18}\text{F}]\text{-NaF}\). Thirty minutes postinjection, the animals were killed by an overdose of pentobarbital and the vasculature was washed with a sterile binding buffer (40 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), and 2.5 mM CaCl\(_2\), pH 7.3) via the left ventricle. Aortic arch was dissected and \([^{18}\text{F}]\text{-NaF}\) uptake was measured \textit{ex vivo} using autoradiography and with a gamma counter (Wallac Wizard, Turku, Finland). Uptake was corrected for injected dose and time.

2.4 | \(\mu\)CT analysis of aortic calcification

\textit{Ex vivo} \(\mu\)CT scans were performed to quantify calcification in the whole aortic tissue (\(n = 11\) for control and DE; \(n = 12\) for warfarin treatment). The paraffin-embedded aortas were scanned for 40/50 minutes by \(\mu\)CT (NanoSPECT/CT, Bioscan, USA) using 45 keV energy, and 360 projections with a resolution of 0.1 mm. Scan data were assessed for Hounsfield units (HU), with HU >1000 considered as positive calcification.

2.5 | Immunohistochemistry

Embedded aortic sections were cut at 4-\(\mu\)m thickness. Plaque size and volume were determined using hematoxylin and eosin staining (\(n = 10\) for control and DE; \(n = 11\) for warfarin treatment). Additionally, plaque characteristics were determined using histochemical analysis for calcification (Alizarin Red S), and elastin fiber breaks (Elastica von Giesson). Stained sections were analyzed by ImageJ Software. Elastin breaks found beneath the atherosclerotic plaque were scored in different sections for every mouse, using a scoring system ranging from 0 to 4: 0, no breaks; 1, one to three breaks; 2, four to six breaks; 3, seven to nine breaks; and 4, >9 breaks. All immunohistochemical scoring were performed blinded to the treatment groups.

The following antibodies were used: anti-uncarboxylated matrix Gla-protein (1:400; IDS, Boldon, UK), anti-bone morphogenetic protein 4 (BMP4; 1:50; Santa Cruz Biotechnology, Dallas, TX), anti-8-hydroxyguanosine (8OHdG; 1:300; Meridian Life Science, TN), anti-MAC3 (1:50, Bioscience, Merck KGaA), and anti-integrin \(\beta\) 3 (ITGB3 1:100, Sigma-Aldrich), Horseradish peroxidase–conjugated secondary antibodies were visualized with Nova-RED substrate (Vector Labs, Amsterdam, Netherlands). Two-dimensional analysis quantification was performed using Leica Application Suite X (Leica Microsystems, Wetzlar, Germany). Data are expressed as the percentage of positive plaque area unless otherwise stated.

2.6 | Cell culture

Human primary VSMCs were derived from tissue explants from males and females aged between 18 and 65 years of age and cultured as described previously.\textsuperscript{26} In brief, VSMCs were cultured in DMEM medium (Gibco, Thermo Fisher Scientific Inc, Paisley, Scotland, UK) supplemented with 20\% fetal bovine serum (FBS), 100 U/ml penicillin and 100 \(\mu\)g/ml streptomycin. Cells were used between passage 4 and 10.

2.7 | Calcification assays

VSMCs were placed in calcifying medium consisting of DMEM supplemented with 2.5\% FBS and 1\% PS in the presence of an additional 1.8 mmol/l calcium. After incubation with stimulus, calcium deposits were assessed by the O-cresolphthalein method and calcium content was normalized for protein content.

2.8 | VSMC proliferation

\textit{xCELLigence} VSMCs were seeded in 96-\textit{xCELLigence}-well. After 24 hours, stimulus was added directly in the well. The slope of impu- dence over 3 days was determined as an indication of proliferation. EdU and VSMCs were seeded in a 24-well plate. The cells were allowed to adhere overnight, then the medium was replaced with stimu- ulus and EdU (5-Ethynyl-2’-deoxyuridine; 10 \(\mu\)M, Life Technologies)
containing 0.5% FBS. After 24 hours, cells were washed twice and fixed using 4% paraformaldehyde (Merck KGaA). Cells were then incubated with staining solution (0.1 M Tris, 1 mM CuSO4, 10.5 µM azide-F488, and 0.1 M ascorbic acid) for 1 hour in the dark. Cells were counterstained with Hoechst (1 µg/ml) for 15 min. Green fluorescent protein and DAPI images were obtained using Cytation 3 Cell Imaging Multi-Mode Reader (BioTek Instruments Inc., Winooski, VT) and % of green fluorescent protein–positive cells over all cells were calculated.

FIGURE 1 Short-term oral anticoagulant effects on atherosclerotic lesions. (A) Schematic overview of experimental setup of the animal experiment. (B) Apoe−/− mice were treated for 6 weeks with WTD without or with warfarin or dabigatran, after which animals were sacrificed. Aortic arches of Apoe−/− mice on WTD without or with warfarin or dabigatran showed no significant difference on plaque size at 6 weeks of treatment. (C) MAC3+ cells were significantly increased in lesions of warfarin-treated mice compared with control (p < .05) and dabigatran (p < .01). (D) Six weeks of warfarin treatment significantly increased ucMGP in atherosclerosis lesions compared to control and dabigatran. a, adventitia; d, aorta descendens; h, heart; i, intima; l, lumen; m, media; ucMGP, uncarboxylated matrix Gla protein; WTD, Western type diet.
2.9 | VSMC migration

VSMC migration was determined using xCELLigence using the Boyden chamber principal. First, VSMCs were starved overnight in 0.5% FBS. The next day, bottom and upper chambers were coated with collagen (0.1 mg/ml collagen G, Biochrome MERCK). Thereafter, 40,000 VSMC were placed in the upper chamber and migration was analyzed over the following 4 hours.

2.10 | Quantitative polymerase chain reaction

RNA extraction, complementary DNA synthesis, and quantitative polymerase chain reaction were performed as described previously. The following primers were used: monocyte chemoattractant protein 1 (MCP1) forward; 5′-CCCCAGTCACCTGCTGTTAT-3′, MCP1 reverse; 5′-TGGAATCCTGAAACCCACTTC-3′, interferon (IFN)-γ forward; 5′-CCAAGCCAATACATGA-3′, IFN-γ reverse; 5′-CTTTTTTCCTTCCGTGTAAA-3′, interleukin (IL)-1β forward; 5′-AACCTCTTCGAGGCAAGGGA-3′, IL-1β reverse; 5′-GTTTACGGGCATGCTTCA-3′, tumor necrosis factor (TNF)-α forward; 5′-TCCACTTTGAGCGTATCGGC-3′, TNF-α reverse; 5′-CGTGTACGAGGATGGATGCAAC-3′, GAPDH forward; 5′-ACGGATTTGGTCGTATTGGGC-3′, GAPDH reverse; 5′-CTTGACGGTGGCATTG3′.

2.11 | Messenger RNA isolation for micro-assay

VSMCs were fasted overnight and stimulated with thrombin, warfarin, or control for 4 hours. We have chosen to compare thrombin and warfarin because both are known to affect VSMC phenotypes relevant for calcification. After 4 hours, VSMCs were washed in ice-cold phosphate-buffered saline before RNA was isolated using the miRNeasy Mini Kit (Qiagen, The Netherlands) including a DNase treatment, according to the manufacturer’s protocols. The concentration of total RNA was measured on a Nanodrop ND-1000 spectrophotometer (Thermofisher, The Netherlands). The integrity of total RNA was checked using RNA Nanochips on a 2100 Bioanalyzer (Agilent Technologies, The Netherlands). Only samples with an RNA Integrity Number > 8.9 were used for microarray analysis.

2.12 | Microarray preparation and data validation

A total amount of 200 μg dye of each sample was used as input material for synthesizing dye (cyanine 3) labeled complementary RNA according to the Agilent one-color Quick-Amp labeling protocol (Agilent Technologies, Amstelveen, The Netherlands). Individual samples were hybridized to an Agilent SurePrint G3 Human Gene Expression 8 × 60 K v2 (Agilent Technologies, Santa Clara, CA). The microarrays were scanned using the Agilent Microarray Scanner (Agilent Technologies, Amstelveen, The Netherlands). Pixel intensities were extracted as raw data from the scan images using Agilent Feature Extraction Software (Agilent). Quantile normalization and data processing were performed using ArrayQC (https://github.com/BigCAT-UM/arrayQC_Module/), a quality control pipeline in R (version 2.10.1; The R Foundation for Statistical Computing, Vienna, Austria). For each spot, the following steps were taken: local background correction, flagging of bad spots, controls, and spots with too low of an intensity, log2 transformation, and quantile normalization. Probes with no flagged bad spots were selected and repeated identifiers merged, resulting in 21,532 transcripts, representing 15,163 unique known genes, and were used for statistical analysis.

2.13 | Selection of differentially expressed genes

Differentially expressed genes were selected using the linear model for microarrays approach. The resulting p values were FWER-corrected using the false discovery rate date method. The following criteria were applied: (1) a false discovery rate–corrected p value <.05 obtained through a moderated t-test, and (2) for the biological replicates an average absolute fold change of 1.5 or higher. Selected gene data sets used “inflammation” and “atherosclerosis” are available on disgenet.

2.14 | Intracellular generation of oxidative stress

To identify intracellular reactive oxygen species (ROS), we used the cell-permeable fluorogenic substrate 2,7-dichlorofluorescein diacetate (Merck KGaA), which is oxidized to 2,7-dichlorofluorescein in the presence of oxidants. VSMCs were seeded in a 96-well plate and left to adhere overnight. Next, cells were incubated with 20 μM 2,7-dichlorofluorescein diacetate for 60 min in the dark at 37°C and 5% CO. After washing, cells were incubated with stimulus in combination with Hoechst (1 μg/ml) and fluorescence intensity was measured (Excitation 485, Emission 529) with Cytation 3 Cell Imaging Multi-Mode Reader (BioTek Instruments Inc., Winooski, VT) for a total of 30 min. Fluorescence intensity was corrected for background measurement and normalized to cell count.

2.15 | Extracellular vesicles quantification

VSMCs were seeded in a 12-well plate and allowed to adhere overnight. Next, VSMCs were stimulated in DMEM 31966, supplemented with 0.5% FBS and 1% PS for 24 hours, after which medium was collected. Medium was centrifuged at 3000g and incubated with CD-63 coupled beads overnight. After washing with phosphate-buffered saline 2% bovine serum albumin, beads were incubated with secondary antibody CD81-APC (1:50; BD Biosciences) and incubated for 60 min in the dark. After washing, exosomes were detected by flow cytometry (BD Accuri C6). Exosome secretion is expressed as arbitrary units, which were calculated as follows: median fluorescence
was multiplied by percentage positive beads and this was normalized for cell number.

2.16 Statistical analysis

All in vitro data were obtained in three or more independent experiments in triplicate (or more) wells. Data are expressed as mean with standard deviation. Data were analyzed using the Mann-Whitney t-test or Kruskal-Wallis one-way analysis of variance using PRISM software (GraphPad 8.2.1, San Diego, CA). Values of \( p < .05 \) were considered statistically significant.

3 RESULTS

Female BL6/C57-Apo–/– mice were fed a WTD (control) or WTD supplemented with VKA (warfarin) or DE for 6 or 18 weeks (Figure 1A). After 18 weeks of treatment, no difference was observed in weight, cholesterol, and triglyceride levels between the groups (Table 1). Increased warfarin and dabigatran plasma levels confirmed respective treatment. Moreover, warfarin significantly increased prothrombin time compared with control, confirming anticoagulant effect (Table 1).

3.1 Effect of short-term oral anticoagulation on atherosclerotic development

Serial sections of the aortic arch were used to visualize atherosclerotic plaques. First, we compared two methods to express plaque size: (1) plaque size based on the section with the largest measured plaque area in mm\(^2\) or (2) plaque size based on volume (mm\(^3\)) determined by reconstructing every fifth section of serially cut specimen. The two methods showed a very strong correlation (\( r = 0.911, p < .0001; \) Figure S1A); thus, because of time, we selected the first method and expressed plaque size in mm\(^2\). Short-term (6 weeks) treatment did not significantly alter plaque size between groups, although warfarin showed a trend toward smaller plaques compared with control and dabigatran treatment (\( p = .24 \) and \( p = .09 \) for warfarin vs control and DE, respectively; Figure 1B). Next, we measured the inflammatory status of plaques by staining macrophage and macrophage-like cells using anti-MAC3 antibodies. Short-term warfarin treatment significantly increased MAC3 positive staining (38.9% of plaque area) compared with both control (25.8% of plaque area) and dabigatran treatment (23.9% of plaque area) (Figure 1C). At 6 weeks of treatment, Alizarin Red S stain did not detect any calcification in any of the groups (data not shown). However, warfarin treatment significantly increased uncarboxylated MGP (ucMGP) in atherosclerotic lesions as compared to both control and dabigatran (\( p < .05; \) Figure 1D). Furthermore, a trend of increased integrin \( \beta3 \) expression was observed after warfarin treatment compared with dabigatran (\( p = .09; \) Figure S1B). No significant difference was observed in smooth muscle cell content and oxidative stress levels (8-OHdG) between the plaques of short-term treatment groups (data not shown).

3.2 Long-term VKA treatment increases active vascular calcification

We next determined long-term (18 weeks of treatment) effects of warfarin treatment on plaque progression and calcification and compared those with control and dabigatran treatment. Warfarin treatment significantly increased atherosclerotic plaque size compared with dabigatran treatment by some 1.7-fold after 18 weeks (0.54 mm\(^2\) and 0.31 mm\(^3\) lesion size, respectively; Figure 2A). Next, the amount of vascular calcification was detected by \( \mu \)CT in whole mouse aortic arches. Warfarin treatment for 18 weeks significantly increased vascular calcification compared to control (30.8 ± 31.9 and 6.22 ± 4.59 total HU for warfarin and control, respectively; \( p < .05 \)) and dabigatran (3.77 ± 6.57 total HU; \( p < .01 \)) (Figure 2B). Dabigatran treatment showed a borderline significant reduction of vascular calcification compared with control (\( p = .07 \)). Second, vascular calcification at 18 weeks was assessed histologically by Alizarin Red S staining of serial sections. Warfarin treatment showed a significant increase in plaque calcification compared with both control and dabigatran treatment (61%, 33%, and 21% of plaque area for warfarin, control, and DE, respectively; Figure 2D). Detection of calcification by \( \mu \)CT and Alizarin Red S showed a strong and significant correlation (\( r = .72, p < .05; \) Figure 2C). To determine whether warfarin induces active calcification, calcification was investigated using the positron emission tomography tracer \(^{18}\text{F}\)-NaF.\(^{15} \) After 18 weeks of treatment, \(^{18}\text{F}\)-NaF uptake by aortas, as measured ex vivo, was significantly increased by warfarin treatment compared with control and dabigatran treatments (Figure 2E). Autoradiography showed that \(^{18}\text{F}\)-NaF activity predominantly localizes to the aortic arch where the atherosclerotic burden is highest (Figure 2F).

To investigate whether calcification was associated with vitamin K deficiency, we determined the correlation between anticoagulation (prothrombin time) and vascular calcification. Increased vascular calcification in the warfarin treated group correlated strongly with prothrombin time after 18 weeks of treatment (\( r = 0.81, p < .01; \) Figure 2G).

**TABLE 1** Model validation of control, warfarin, and dabigatran after 18 weeks of treatment

|                  | Control      | Warfarin    | Dabigatran |
|------------------|--------------|-------------|------------|
| Weight (g)       | 23.82 ± 1.60 | 22.50 ± 1.68 | 24.33 ± 2.46 |
| Cholesterol (mM) | 21.27 ± 3.34 | 21.54 ± 3.07 | 18.93 ± 1.74 |
| Triglycerides (mM) | 2.77 ± 0.20 | 2.96 ± 0.28 | 2.70 ± 0.17 |
| Dabigatran (ng/ ml) | –           | –           | 370.3 ± 175.9 |
| Warfarin (mg/l)  | –           | 11.52 ± 5.81 | –          |
| Prothrombin time (s) | 8.73 ± 0.83 | 54.2 ± 22.7* | 12.5 ± 4.28 |

\( ^* p < .05 \)
FIGURE 2 Long-term oral anticoagulant treatment has a differential effect on vascular calcification. Apoe$^{-/-}$ mice were treated for 18 weeks with WTD without or with warfarin or dabigatran, after which animals were sacrificed. (A) Aortic plaque size was significantly increased after 18 weeks of warfarin treatment compared with dabigatran treatment ($p < .05$). (B) Vascular calcification detected by µCT on whole aortic arches showed significant increased calcification after 18 weeks of warfarin treatment compared with control ($p < .05$) and dabigatran ($p < .01$). (C) Calcification detected by µCT and Alizarin Red S strongly correlate ($r = 0.72$, $p < .05$). (D) Calcification in atherosclerotic lesions was visualized by Alizarin Red S and was significantly increased after warfarin treatment compared with control ($p < .05$) and dabigatran ($p < .01$). (E) $^{18}$F-NaF activity in whole aortic arches was significantly increased after 18 weeks warfarin treatment compared with control and dabigatran ($p < .05$). (F) Visualization of $^{18}$F-NaF in an aortic arch by autoradiography. (G) In warfarin-treated mice, amount of vascular calcification correlates significantly with anticoagulation as measured by elevated prothrombin time ($r = 0.81$, $p < .01$). d, aorta descendens; h, heart; l, lumen; WTD, Western type diet
3.3  Differential effects of oral anticoagulation on atherogenesis

Vascular calcification is also linked to plaque progression and used in the clinic to determine cardiovascular risk. Therefore, we calculated the increase in plaque progression as an x-fold increase in plaque size from 6 to 18 weeks of treatment. Warfarin treatment showed a significant 7.2-fold increase in plaque progression compared with a 3.7-fold increase in control (p < .05) and a 1.9-fold increase in dabigatran treatment (p < .001). Moreover, dabigatran treatment significantly reduced plaque progression compared with control (p < .01; Figure 3A). To further test whether calcification is related to vascular vitamin K deficiency, ucMGP positivity in the plaques was analyzed. Warfarin significantly increased ucMGP in atherosclerotic plaques (0.89% of plaque area) compared with control (0.09% of plaque area; p < .001) and dabigatran (0.38% of plaque area; p < .05) (Figure 3B). UcMGP positivity was also significantly increased in dabigatran compared with control treatment (Figure 3B). To examine the contribution of MGP in the process of calcification we correlated ucMGP presence with vascular calcification detected with Alizarin Red S. UcMGP significantly correlates with vascular calcification in atherosclerotic plaques (r = 0.58, p < .01; Figure S1C). Moreover, effects of increased ucMGP in atherosclerotic plaques was determined by assessing BMP4, a known downstream effector and promoter of osteochondrogenic differentiation. Similar to ucMGP, warfarin treatment significantly increased BMP4 in the atherosclerotic plaque compared with both control and dabigatran (Figure 3C). In contrast to ucMGP, no difference was observed between dabigatran and control.

3.4  Long-term VKA treatment induces a vulnerable atherosclerotic plaque phenotype

Next, plaque composition was determined to gain insight into plaque vulnerability. Elastin breaks in the tunica media underneath the atherosclerotic plaque were significantly more abundant after warfarin treatment (score 2.3) compared with control (score 1.5) or dabigatran treatment (score 1.3) (p < .05; Figure 3D). Oxidative stress is considered a major cause of vascular calcification. Oxidative stress, measured by 8OHdG+ cells, was significantly increased after 18 weeks warfarin treatment compared with control and dabigatran (Figure 3E). No difference was observed in smooth muscle cell content, macrophages (MAC3), matrix metalloproteinases 2 and 9, or necrotic core size between any of the groups (data not shown).

3.5  Systemic inflammation is not affected by warfarin or dabigatran treatment

After 18 weeks of treatment, cytokine levels were determined in plasma to investigate whether systemic inflammation in involved in OAC effects on atherosclerosis. No difference in cytokines levels were found between any of the treatment regimens (Table S1).

4 | WARFARIN PROMOTES AN INFLAMMATORY RESPONSE IN VASCULAR SMOOTH MUSCLE CELLS

Because warfarin treatment induced a proinflammatory phenotype at 6 weeks of treatment, we stimulated VSMC with warfarin. Proliferation and migration of VSMC is a key feature in the genesis of atherosclerosis. Unexpectedly, warfarin treatment reduced proliferation to 68% compared with control as measured by xCEL-Ligence (Figure 4A). Reduced VSMC proliferation by warfarin was confirmed by lower EdU incorporation in a dose-dependent manner (p < .001; Figure 4B). Moreover, warfarin significantly reduced VSMC migration compared with control (Figure 4C). Because warfarin treatment for 6 weeks increased MAC3+ cells in the atherosclerotic plaque, we measured effects of warfarin on VSMC inflammation. VSMC treated with warfarin for 4 hours significantly increased MCP1 (1.6-fold; Figure 4D), IFN-γ (4.3-fold; Figure 4E), and IL-1β (2-fold; Figure 4F) messenger RNA levels. Although not significant, warfarin treatment also increased TNF-α messenger RNA levels (Figure 4G).

4.1  Interaction of warfarin but not dabigatran with oxidized low-density lipoprotein

The experimental animal model used to investigate the effect of oral anticoagulation was the BL6/C57-Apoe−/−, which is known for its increased cholesterol levels. We tested potential synergistic effects of warfarin or dabigatran in combination with oxidized low-density lipoprotein (oxLDL). Although oxLDL alone was a significant inducer of VSMC calcification (4.5- to 6.4-fold compared with control), no effect of additional dabigatran on VSMC calcification was observed (Figure 5A). Moreover, dabigatran alone did not influence VSMC calcification (Figure 5A). On the other hand, warfarin treatment alone significantly increased VSMC calcification (2-fold; Figure 5B). Co-stimulation of warfarin with oxLDL significantly increased VSMC calcification compared with either warfarin or oxLDL alone (2.2-fold compared with oxLDL alone; Figure 5B).

4.2  Thrombin increased oxidative stress and secretion of extracellular vesicles

Because warfarin increased oxidative stress in Apoe−/− mice, we tested whether warfarin and dabigatran also induce oxidative stress in VSMCs in vitro. Intracellular oxidative stress in VSMCs was determined over 30 minutes, directly after stimulation with either warfarin, thrombin, or thrombin combined with dabigatran. Warfarin significantly increased oxidative stress compared with control (50 µM warfarin, 3.7-fold increase; Figure 5C). Thrombin significantly increased oxidative stress in VSMCs compared with vehicle treatment (1.9-fold; Figure 5C). Co-stimulation of dabigatran with
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Thrombin significantly reduced thrombin induced oxidative stress ($p < .05$; Figure 5C). Dabigatran stimulation alone had no effect on oxidative stress. Oxidative stress is an inducer of extracellular vesicle secretions, which are known to induce VSMC calcification. Therefore, we analyzed the number of secreted extracellular vesicles from VSMCs. Warfarin stimulation significantly increased extracellular vesicles compared with control (2.2-fold, $p < .01$; Figure 5D). Thrombin also increased extracellular vesicle release significantly (3.1-fold), which was dose dependently reduced after co-stimulation with dabigatran (2-fold for 0.1 $\mu$M dabigatran and 1.5-fold for 1 $\mu$M dabigatran compared with control; Figure 5D). Moreover, dabigatran stimulation alone significantly reduced the number of extracellular vesicles compared with control (0.9-fold of control for 1 $\mu$M dabigatran; Figure 5D).

4.3 | Differently expressed genes after thrombin and warfarin stimulation in VSMCs

To further analyze the effects of warfarin and thrombin on VSMCs, we performed transcriptomic analysis after 4 hours of stimulation of VSMCs with warfarin and thrombin compared with vehicle control. Although warfarin affected 44 transcripts (27 genes increased expression and 17 reduced compared with control), thrombin altered a total of 1582 transcripts (expression of 979 genes were increased and expression of 603 genes were reduced compared with control). Altered genes were compared with known datasets associated with atherosclerosis development (1133 genes; Table 2). Thrombin significantly changed expression of 107 genes in this transcriptome whereas warfarin only significantly altered expression of four genes.
FABP5 and IL1b were significantly altered for both warfarin and thrombin. Transcriptomic analysis was also compared with a known inflammatory gene dataset (428 genes; Table 3) because of the important role of inflammation in atherosclerosis together with the observed inflammatory effect of warfarin both in vivo and in vitro. Here, thrombin altered 43 genes, of which only three were also altered by warfarin stimulation (IL-1b, leukemia inhibitory factor, CXCL2).

5 | DISCUSSION

In the presented study, we demonstrate that warfarin and dabigatran have differential effects on atherosclerosis. Warfarin treatment accelerated plaque inflammation, oxidative stress, calcification activity, and plaque progression. By contrast dabigatran had no effect on vascular inflammation or calcification and reduced plaque progression in vivo. These data suggest that the choice of oral anticoagulant treatment in patients at risk of venous or arterial thrombosis may have important effects on atherosclerotic disease, indicating that further detailed clinical studies are now required.

Warfarin promotes atherosclerotic disease activity by increased presence of calcification and calcification activity. Recently, greater calcification volume—but not higher density—was associated with increased cardiovascular disease risk. Our data show that warfarin increases calcification volume in atherosclerotic plaques, suggesting that warfarin increases plaque vulnerability. Dabigatran had no effect on calcification and even reduced plaque progression, implying that local effects play key roles in cardiovascular disease development. It is known that warfarin, as with all VKAs, inhibits carboxylation of
vitamin K–dependent proteins thereby reducing coagulation tendency at the expense of MGP activity. MGP is one of the strongest vascular calcification inhibitors and reduced activation of MGP by warfarin appears to tip the balance towards calcification. Carboxylated MGP binds to BMP4, thereby reducing osteochondrogenic differentiation. We confirm that warfarin treatment increased the presence of BMP4.

| TABLE 2 | Altered gene transcripts of an atherosclerotic dataset of genes |
| --- | --- |
| Gene | Thrombin LogFC | Warfarin LogFC | Gene | Thrombin LogFC | Warfarin LogFC | Gene | Thrombin LogFC | Warfarin LogFC |
| ABCA1 | -1.29 | 0.02 | GREM1 | 2.15 | 0.20 | PLA2G4A | 0.67 | 0.11 |
| ABR | 0.64 | 0.18 | HAS2 | 2.00 | 0.44 | PLAT | 0.67 | 0.14 |
| ADAM8 | -0.75 | -0.08 | HILPDA | 0.88 | -0.06 | PLAU | 1.52 | 0.05 |
| ADAMTS1 | 0.66 | -0.18 | HMGAI | 0.62 | 0.09 | PNP | 0.96 | 0.04 |
| ADAMTS5 | -2.08 | -0.08 | HS3ST1 | 1.28 | 0.12 | PPARG | 1.28 | 0.09 |
| AHRR | 0.01 (NS) | 0.78 * | HSD11B1 | 0.62 | 0.12 | PTGS1 | 0.62 | 0.19 |
| APOL6 | -0.83 | -0.03 | HSPA2 | 1.95 | -0.16 | PTK2B | -0.88 | -0.12 |
| BDNF | -0.60 | -0.34 | ICAM1 | 0.83 | 0.24 | RGS2 | 0.73 | -0.14 |
| BMP4 | -0.74 | -0.16 | IL11 | 0.60 | -0.04 | SAMD4A | 1.45 | 0.01 |
| CASP1 | -0.71 | 0.18 | IL1B | 1.30 | 0.76 | SAMD9 | 0.79 | 0.05 |
| CASP3 | 0.73 | -0.06 | IL33 | 0.83 | 0.05 | SERPINE1 | 1.69 | -0.12 |
| CAV1 | 0.85 | 0.06 | IL6 | 0.76 | -0.25 | SLC16A3 | 0.94 | -0.16 |
| CCL2 | 1.47 | 0.24 | IRS2 | -0.81 | -0.04 | SLC17A5 | 0.79 | -0.14 |
| CD44 | 0.67 | 0.17 | ISG20 | 1.46 | 0.09 | SLC6A4 | -0.67 | 0.09 |
| CDKN1C | -0.82 | -0.15 | ITIH4 | -0.70 | 0.13 | SMAD3 | 0.78 | 0.00 |
| CHDH | 1.53 | -0.12 | KLF4 | 0.88 | -0.22 | SOCS1 | 0.65 | 0.37 |
| CREB3 | 0.60 | -0.09 | KLF5 | -0.72 | -0.32 | SOCS3 | 0.78 | 0.10 |
| CRY1 | 0.99 | 0.06 | LDB2 | -1.19 | 0.06 | SOD2 | 0.99 | 0.04 |
| CTF1 | -0.83 | -0.08 | LIPE | -0.70 | -0.06 | ST8SIA1 | -1.56 | 0.08 |
| CXCL1 | 2.49 | 0.41 | LMNA | 0.95 | -0.08 | TCF7L2 | -0.91 | -0.38 |
| DDAH1 | 1.45 | -0.25 | LRP8 | 0.67 | 0.26 | TFPI2 | 0.97 | 0.08 |
| DUSP1 | 0.76 | -0.29 | MAP2 K1 | 0.89 | -0.05 | THBD | 2.71 | 0.19 |
| ECE2 | 0.71 | -0.09 | MAP3 K5 | -1.17 | 0.16 | TNFRSF11B | 0.76 | -0.23 |
| EDN1 | -1.25 | -0.20 | MPRIP | 0.89 | -0.19 | TNFRSF12A | 1.03 | -0.39 |
| EDNRA | -0.99 | -0.32 | MTSS1 | 1.81 | 0.23 | TNFRSF14 | -0.72 | 0.14 |
| EFNB1 | -0.77 | -0.17 | MYH9 | 0.63 | -0.28 | TNFRSF4 | 0.29 (NS) | 1.22 * |
| EGFR | 0.78 | 0.08 | NCEH1 | 0.71 | 0.21 | TNFSF4 | 1.10 | -0.05 |
| ELK1 | 0.61 | 0.14 | NOTCH1 | 0.78 | 0.21 | TNFSF9 | 0.66 | 0.01 |
| ENPP1 | 0.63 | -0.12 | NOV | 0.69 | -0.09 | TRAF3IP2 | 0.88 | 0.24 |
| ETS1 | 0.68 | 0.02 | NR1D1 | -0.82 | 0.10 | TRIB3 | -1.57 | -0.05 |
| FABP5 | -0.70 | 0.60 * | NR4A3 | 3.41 | 0.18 | UNC5B | -0.76 | -0.05 |
| FGF2 | -0.96 | -0.25 | NRG1 | -1.47 | -0.16 | VCL | 1.05 | -0.15 |
| FGF1 | 0.60 | 0.00 | NUP62 | 0.69 | -0.13 | VKORC1 | 0.61 | 0.01 |
| FOS | -1.00 | -0.23 | PDGFA | 1.50 | -0.09 | VWF | 1.72 | 0.21 |
| FST | 0.95 | -0.28 | PECAM1 | 1.12 | 0.15 | WNT5A | -0.69 | 0.08 |
| FURIN | 0.87 | 0.20 | PIK3CD | 1.15 | 0.00 | XPR1 | 0.94 | -0.20 |

Increased (red) and reduction (blue) compared with control. All thrombin, except for (NS), altered gene expressions were significant different compared with control and *present significant difference of warfarin compared with control. FC, fold change; NS, non significant.
in the atherosclerotic plaque. Unexpectedly, dabigatran increased ucMGP presence in atherosclerotic plaque, but in contrast to warfarin did not affect BMP4 presence in atherosclerotic plaque. This suggests that in dabigatran-treated animals elevated levels of ucMGP point toward a coping mechanism for elevated calcification pressure being part of increased total MGP.

Carboxylated MGP binds to calcium crystals and extracellular vesicles, blocking further growth of calcification. Warfarin treatment increased ucMGP already in early atherosclerotic lesions, even before calcification could be visualized. Active calcification, detected by $[^{18}F]$-NaF is associated with increased plaque vulnerability. We show that mice treated with warfarin displayed higher $[^{18}F]$-NaF positivity in the vasculature compared with dabigatran and control, indicating that VKAs induce active mineralization. Our data are in line with recently published preclinical studies and clinical studies comparing VKA, NOAC, or no oral anticoagulant treatment on vascular calcification. VKA treatment showed higher prevalence of vascular and valvular calcification, which was not seen in NOAC-treated patients. Moreover, plaque burden and prevalence of high-risk plaques have been shown to be increased in patients using VKA compared with NOAC treatment, although caution should be taken with generalizing NOAC treatments.

Oxidative stress has been shown to increase vascular calcification development. Warfarin increases oxidative stress in vivo and in vitro, whereas dabigatran reduced thrombin-induced oxidative stress. VKAs have been put forward to induce oxidative stress via inhibition of the vitamin K cycle. VKAs are also known to interact with cytochrome P450 (CYP) and our transcriptomic data show elevated CYP1B1 transcript after warfarin treatment. CYP1B1 has been shown to increase oxidative stress in VSMCs. Dabigatran, on the other hand, has been shown to block oxidative stress in mice thereby improving nitric oxide synthesis. Extracellular vesicles from VSMCs play an important role in the initiation of calcification, and both warfarin and thrombin increased extracellular vesicle secretion. Taken together, warfarin induces impaired carboxylation of MGP, and increases oxidative stress and extracellular vesicle release, thereby supporting vascular calcification. In contrast, dabigatran inhibits the detrimental side effects of thrombin on VSMCs.

Both thrombin and warfarin increased IL-1ß transcripts, which has been associated with exacerbation of atherosclerosis.

### Table 3: Altered gene transcripts of an inflammatory dataset

| Gene   | Thrombin LogFC | Thrombin logFC | Warfarin LogFC | Warfarin logFC |
|--------|----------------|----------------|----------------|----------------|
| AQP3   | 0.90           | 0.08           | -1.47          | -0.16          |
| BDKRB1 | 0.64           | 0.20           | 1.50           | -0.09          |
| BTN1   | -0.60          | -0.34          | 1.12           | 0.15           |
| BMP4   | -0.74          | -0.16          | 0.67           | 0.14           |
| CASP1  | -0.71          | 0.18           | 1.52           | 0.05           |
| CCL2   | 1.47           | 0.24           | 1.28           | 0.09           |
| CD44   | 0.67           | 0.17           | 1.69           | -0.12          |
| CD83   | 0.63           | 0.40           | 0.66           | 0.01           |
| CXCL1  | 2.49           | 0.41           | 0.78           | 0.00           |
| CXCL2  | 1.68           | 1.04 *         | 0.65           | 0.37           |
| CXCL6  | 1.55           | 0.47           | 0.78           | 0.10           |
| EDN1   | -1.25          | -0.20          | 0.97           | 0.08           |
| EFNB1  | -0.77          | 0.17           | 2.71           | 0.19           |
| FGF2   | -0.96          | -0.25          | 0.65           | 0.12           |
| ICAM1  | 0.83           | 0.24           | 1.70           | -0.03          |
| IL1B   | 1.30           | 0.76 *         | 0.76           | -0.23          |
| IL1RAP | -1.19          | 0.13           | 1.03           | -0.39          |
| IL33   | 0.83           | 0.05           | -1.04          | -0.01          |
| IL4R   | 0.74           | 0.12           | 1.72           | 0.21           |
| IL6    | 0.76           | -0.25          | 0.90           | -0.08          |
| LIA5   | -0.61          | -0.02          | 0.73           | -0.06          |
| LIF    | 1.65           | 0.75 *         | 1.70           | -0.03          |

Increased (red) and reduction (blue) compared with control. All thrombin altered gene expressions were significant different compared with control and *present significant difference of warfarin compared with control. FC, fold change.
Microcalcification can initiate inflammation and inflammation can promote calcification. We show increased presence of macrophages in early atherosclerotic lesions and elevated inflammatory markers in VSMCs after warfarin treatment. Moreover, we demonstrate that warfarin treatment increases \( ^{[18]} \text{F}-\text{NaF} \) uptake, which is indicative of presence of microcalcification. Warfarin has previously been shown to have immunomodulatory effects, including promoting an inflammatory environment. It has been demonstrated that calcified VSMCs initiate macrophage migration and promote a pro-inflammatory environment. In early atherosclerotic lesions, hydroxyapatite nanocrystals are present, associated with initial mineralization. Additionally, calcium crystals activate macrophages thereby promoting a pro-inflammatory environment and gaining osteogenic activity. Consequently, inflammatory macrophages promote calcification via release of TNF-\( \alpha \). In line with these data, we confirm that mice treated with warfarin had more macrophages in early atherosclerotic lesions. In conclusion, macrophage rich plaques display increased osteogenic activity, further enhancing the strong correlation between inflammation and vascular calcification.

Thrombin can affect atherosclerosis development by promoting inflammation via cytokine production, proliferation and cytoskeletal rearrangement of VSMC, fibroblast stimulation and nitric oxide synthesis. In atherosclerosis, thrombin also promotes leukocyte recruitment, whereas reduction of thrombin by approximately 50% reduced macrophages content. Here, we show that dabigatran reduced atherogenesis and that thrombin inhibition induced oxidative stress and extracellular vesicle secretion in vitro. Moreover, thrombin stimulation in VSMCs increased a wide range of transcript genes associated with atherosclerosis and inflammation. Thrombin binds and cleaves the N-terminal exodomain of PAR1, thereby initiating PAR1 signaling. Dabigatran inhibits thrombin, thereby attenuating thrombin-induced PAR signaling resulting in diminished or altered atherosclerosis. Indeed, dabigatran has been shown to reduce thrombin induced inflammatory response. Moreover, it has been shown that dabigatran increases bone turnover and reduces bone mineralization compared with warfarin treatment. Finally, edoxaban, a direct FXa inhibitor, has recently been shown to inhibit mal-adaptive vascular remodeling compared with warfarin treatment, in line with our findings.

This study has several limitations. The atherosclerotic Apoe<sup>-/-</sup> mice model is a widely accepted animal model for studying atherosclerosis. Although there are great similarities in morphology of plaques compared with humans, atherosclerotic plaques in mice will not rupture. Therefore, potential consequences of myocardial infarction or stroke are absent in this model. In this study, we achieved dabigatran plasma levels of 370 ng/ml (Table 1), which is higher compared with patients with concentrations varying between 50 and 180 (trough-peak). However, murine thrombin has lower affinity to dabigatran than human thrombin. Thus, increased dabigatran concentration in our model is roughly comparable to patient concentrations. Moreover, our dosage of warfarin in mice (e.g., 3 mg/g and mice eat approximately 3 g per day, daily intake of 9 mg per day) exceeded dose in humans (5 mg per day/70 kg). Nevertheless, warfarin was combined with elevated vitamin K1 to prevent bleeding and was used to study VKA effects on the vessel wall. In the present study, we determined the differential effects of warfarin and dabigatran on the vessel wall and possible consequence in atherosclerosis development.

Our data are of clinical importance because many patients are treated with cholesterol-lowering drugs such as statins, known to also impact the vasculature. Statins have been shown to reduce atherosclerotic plaque vulnerability and subsequent cardiovascular morbidity and mortality. Combination therapy of statin treatment with either warfarin or NOAC might impact treatment efficacy and interactions between medication needs to be investigated in future clinical trials.

In conclusion, VKA aggravates atherogenesis via its direct effects on VSMCs. Our data show that VKA increases atherosclerotic disease activity, increasing plaque inflammation, oxidative stress, and calcification activity; effects that translate into faster plaque progression. In contrast, these effects are not observed with dabigatran, which appeared to have a beneficial effect on disease progression. Our findings suggest that the choice of anticoagulant in patients at risk of arterial or venous thrombosis may have important off-target effects on bystander atherosclerotic disease, with further studies in humans now required.

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**CONFLICT OF INTEREST**

Rich H. van Gorp is employed by NattoPharma. Joanne van Ryn is employed by Boehringer Ingelheim. Leon J. Schurgers received research funding from Boehringer Ingelheim related to this work and from NattoPharma, Bayer, Daichii-Sanky, and IDS not related to this work. Felix Mottaghay is on the advisory board of Advanced Accelerator Applications GmbH and has received institutional grants from GE Healthcare, Siemens, and Nano-Map not related to this work. Herni M. H. Spronk received funding for research from Bayer and Pfizer outside this work. Henri M. H. Spronk and Leon J. Schurgers are stockholders in Coagulation Profile. All other authors declare no conflict of interest.

**AUTHOR CONTRIBUTIONS**

Rick H. van Gorp, Vanessa Bröker, Matthijs Bauwens, and Peter Leenders conducted the animal experiments. Peter Leenders performed the experimental surgery. Rick H. van Gorp and Peter Leenders performed the histological analysis. Danyel Jennen and Jaco J. Briedé performed microarrays and data validation.
Rick H. van Gorp, Joanne van Ryn, Henri M. H. Spronk, Chris P. Reutelingsperger, and Leon J. Schurgers designed and prepared the study. Ingrid Dijkgraaf, Marc R. Dweck, Jan Bucerus, Vincent Brandenberg, and Felix Mottaghy analyzed and interpreted vascular calcification data. Rick H. van Gorp and Vanessa Bröker performed in vitro experiments. Rick H. van Gorp, Chris P. Reutelingsperger, and Leon J. Schurgers wrote the manuscript. Joanne van Ryn and Henri M. H. Spronk proofread the manuscript. Chris P. Reutelingsperger, and Leon J. Schurgers performed final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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