DR MARCELLO RATTAZZI (Orcid ID : 0000-0003-3437-7263)

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Warfarin, but not Rivaroxaban, promotes the calcification of the aortic valve in ApoE-/- mice

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Marcello Rattazzi[1,2], Elisabetta Faggin[1], Elisa Bertacco[1], Chiara Nardin[1], Leopoldo Pagliani[3], Mario Plebani[1], Francesco Cinetto[1,2], Diego Guidolin[4], Massimo Puato[1], Paolo Pauletto[3].

Affiliations:

1) Department of Medicine - DIMED, University of Padova, Italy

2) Medicina Generale I^, Ca' Foncello Hospital, Treviso, Italy

3) ORAS Rehabilitation Hospital, Motta di Livenza, Treviso, Italy

4) Department of Neuroscience, University of Padova, Italy

Corresponding author:

Marcello Rattazzi MD, PhD

Department of Medicine - DIMED, University of Padova, Medicina Interna I^, Ca' Foncello University Hospital, Via Ospedale 1, 31100 Treviso, Italy.

phone: +390498211867- +390422322207, fax:+390498754179,

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e-mail: marcello.rattazzi@unipd.it; marcello.rattazzi@aulss2.veneto.it

ABSTRACT

Introduction: Vitamin K antagonists, such as warfarin, are known to promote arterial calcification through blockade of gamma-carboxylation of Matrix-Gla-Protein. It is currently unknown whether other oral anticoagulants such as direct inhibitors of Factor Xa can have protective effects on the progression of aortic valve calcification.

Aims: to compare the effect of warfarin and rivaroxaban on the progression of aortic valve calcification in atherosclerotic mice.

Results: 42 ApoE-/- mice fed with Western-type Diet (WTD) were randomized to treatment with warfarin (n=14), rivaroxaban (n=14) or control (n=14) for 8 weeks. Histological analyses were performed to quantify the calcification of aortic valve leaflets and the development of atherosclerosis. The analyses showed a significant increase in valve calcification in mice treated with warfarin as compared to WTD alone (p= 0.025) or rivaroxaban (p=0.005), whereas no significant differences were found between rivaroxaban and WTD (p=0.35). Quantification of atherosclerosis and intimal calcification was performed on the innominate artery of the mice and no differences were found between the three treatments as far as atherogenesis and calcium deposition is concerned. In vitro experiments performed by using bovine interstitial valve cells (VIC) showed that treatment with rivaroxaban did not prevent the osteogenic conversion of the cells but reduce the over-expression of COX-2 induced by inflammatory mediators.

Conclusion: We showed that warfarin, but not rivaroxaban, could induce calcific valve degeneration in a mouse model of atherosclerosis. Both the treatments did not significantly affect the progression of atherosclerosis. Overall, these data suggest a safer profile of rivaroxaban on the risk of cardiovascular disease progression.

Keywords: aortic valve stenosis, atherosclerosis, vascular calcification, warfarin, rivaroxaban, ApoE-/- mice.

Introduction

Vascular calcification is a degenerative phenomenon commonly observed during atherogenesis and aortic valve calcification. A number of convincing evidence showed that, independently from the vascular site (intima, media layer or valve leaflet), the deposition of calcium should be now considered a tightly regulated biological process. This process involves a series of mediators/pathways acting either as promoters or inhibitors of the calcification process.(1) Cell types potentially involved in calcium deposition include vascular smooth muscle cells (VSMC), interstitial valve cells (VIC), circulating osteoprogenitor cells and mesenchymal pluripotent cells resident in the vascular tissue.(1-4) The presence of ossified bone/cartilaginous metaplasia and the expression of osteogenic cell makers within the pathological arterial wall have been reported by several studies conducted both in animal models of atherosclerosis and humans samples.(1, 5) These findings suggest that during atherogenesis/aortic valve calcification some cellular elements of different origin can acquire an osteogenic phenotype and recapitulate some of the processes driving calcium deposition in the bone. We and others previously demonstrated that under certain pathologic conditions (such as hyperphosphatemia, oxidative stress, and inflammation) VIC can differentiate into calcifying cells and promote the mineralization of collagen matrix.(4, 6, 7) In this context, it has been showed that Matrix-Gla-Protein (MGP) is a potent inhibitor of vascular calcification, preventing both the crystal complexs nucleation and the Bone Morphogenic Protein-2 (BMP-2) induced osteogenic differentiation of vascular cells.(8) Of note, previous animal studies showed that the treatment with the vitamin K antagonist (VKA) warfarin might exert detrimental effects on both vascular and valve calcification, mainly through the inhibition of MGP carboxylation. (9, 10) In fact, only the carboxylated form of MGP (c-MGP) is protective towards calcium deposition whereas the uncarboxylated (uc-MGP) is ineffective in protecting the vascular tissue from mineralization.(11, 12) The blockade of c-MGP generation in patients treated with warfarin can explain the association observed between the use of this drug and the risk of vascular calcification, including the progression of aortic valve disease.(9)

Rivaroxaban is a direct inhibitor of Factor Xa (FXa), a serine endopeptidase that plays a central role in the coagulation cascade by catalyzing the conversion of prothrombin to thrombin on vascular cell surface. Interestingly, Factor Xa has been implicated in signalling events on different cell types, including VSMC and fibroblasts. In particular FXa has been shown to act as chemoattractant for fibroblasts, mitogen for VSMC and inducer of inflammatory activation and senesce of endothelial cells.(13-16) All these findings underscore a possible contribution for FXa inhibition with rivaroxaban in the prevention of atherosclerotic disease progression, although in vivo studies so far yielded conflicting results.(17, 18) To date no information are available about the role of rivaroxaban during the progression of vascular calcification. In particular, it is unknown whether rivaroxaban can have protective effects on the progression of aortic valve calcification. To this purpose we performed an in vivo study on atherosclerotic mice (ApoE -/- mice) to compare the effect of warfarin and rivaroxaban on the development of both atherosclerotic and valvular calcification.

Material and methods

Animals

Starting from 8 weeks of age, 42 ApoE-/- mice purchased from Charles Rivers, were fed with irradiated vitamin K-deficient Western-Type Diet (WTD), supplemented with Vitamin K1 (1.5 mg/g food) (Mucedola Srl, Italy) for 8 weeks. At the end of this period the mice were randomized in three groups (n=14, with 7 male and 7 female for each group) receiving the WTD containing Vitamin K1 supplemented with one of the following treatments: 5 mg rivaroxaban/kg bodyweight/day (Bayer), 3 mg VKA warfarin (V/K1 diet) or control. The V/K1 diet has been developed to study the effects of VKA on the metabolism of Gla-proteins synthetized outside of the liver. In fact, the supplementation with Vitamin K1 overcome the poisoning effects of VKA on the synthesis of coagulation factors, but does not affect the inhibition of γ-carboxylation of proteins produced by extrahepatic tissues, including the arterial vessels. (19, 20)

At the end of the treatment period the animals were euthanized and the vascular tissues were collected for the subsequent analyses. In particular, the aortic sinus and the innominate artery of each animal were isolated, embedded in OCT (Sakura) and frozen in liquid nitrogen. The aortas were cleaned and immediately snap frozen in liquid nitrogen. All the samples were then stored at -80°C until analysis.

All of the procedures were approved by the local ethics committee and the Italian Ministry of Health and were conducted according to the National Institutes of Health Principles of Laboratory Animal Care.

Morphometric analysis

Histological studies were performed on 10 μm serial crysections of both aortic sinus and innominate artery. In particular, for each animal approximately a total of twelve cryosections were selected (three sections on four different levels spaced 200 μm apart). The image acquisition was performed by using a Leica microscope assembled with a DFC 420 camera.

Each section was stained by using a standard von Kossa procedure to identify calcium-phosphate deposits within both the aortic valve leaflets and the atherosclerotic lesions. The quantification of valve/intimal calcification was performed trough a dedicated image analysis protocol developed by using ImageJ (NIH). In particular, for each section we measured both the total area occupied by the valve leaflets and the percentage of calcified area within the tissue. A similar approach was used to estimate in the same section of the innominate artery both the extension of the atherosclerotic lesions and the intimal area occupied by calcium deposits. Measurements obtained in the twelve sections of the aortic sinus and the innominate artery of each animal were then averaged and subjected to statistical analyses.

In vitro analysis

For the in vitro studies we used primary interstitial valve cells (VICs) obtained from aortic bovine valve leaflets by culture of tissue fragments digested with type-I collagenase, elastase, and soybean trypsin inhibitor (Sigma, St. Louis, MO, USA). (4) The cells were expanded in DMEM containing 4.5 g/L glucose plus FBS 20%, 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma, St. Louis, MO, USA). Starting from the mixed population a cloning procedure, based on a limiting dilution technique, was performed to isolate several clones of VIC as previously described. (4) For the present study we used a selected clonal cells population carrying a strong pro-calcific potential.(6, 21) Selected clonal cells were seeded at a density of 10^4cellule/cm^2 in 6-well plates and cultured in DMEM supplemented with 5% FBS. The cells were treated for 12 days in DMEM + 5% FBS supplemented with endotoxin (LPS 500 ng/ml, Sigma, St. Louis, MO, USA) to promote the osteogenic differentiation with or without the addition of rivaroxaban diluted in DMSO (30 µg/ml). Media and treatments were renewed every third day. For total RNA extraction we performed in parallel three technical and two biological replicate for each condition.

Gene expression analysis

At the end of treatment, total RNA was isolated by using a protocol with Trizol (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer' instructions. RNA purity and concentration were evaluated by spectrophotometry using NanoDrop ND-2000 (Thermo Fisher Scientific, Wilmington, DE). To assess the effects of rivaroxaban on the gene expression of osteogenic and inflammatory markers the levels of alkaline phosphatase (ALP) and cyclooxygenase-2 (COX-2) mRNA were examined by using real-time PCR.

1 µg of total RNA was retrotrascribed with iScript Reverse Transcription Supermix kit (Bio-Rad Laboratories, CA, USA). The qPCR was performed using SsoFast Eva Green Supermix (Bio-Rad Laboratories, CA, USA) with a CFX96 thermocycler (Bio-Rad). Each sample was run in triplicate. The sequences of primers used in real-time PCR are shown in the supplemental table. Gene expression

was calculated by the comparative Ct (2−ΔΔCt) method: each sample was quantified against its bovine hydroxymethylbilane synthase (HMBS) and normalized to the control group. The results are presented as mean fold increase ± SEM.

Statistical analysis

Comparisons between the three groups for both aortic valve calcification and atherosclerosis development have been performed by using ANOVA followed by LSD post-hoc analysis. A similar approach was used to compare the in vitro results of gene expression analyses. Statistical significance was considered for $p<0.05$.

Results

Animal study

After 8 weeks of WTD 42 ApoE-/- mice were randomized to receive a diet supplemented with warfarin, rivaroxaban or control. At the end of the treatment period (8 weeks) vascular and valve tissues were collected for histological analyses. At the sacrifice we did not observed significant difference between the three groups in term of body weight, plasma lipids and calcium/phosphate levels (data not shown). The morphometric analysis was performed on cryosections of the aortic sinus to estimate the accumulation of calcium within the valve leaflets. In particular, the percentage of valve area positive for von Kossa staining was estimated in each group of animals. The analysis showed a significant increase in calcium accumulation within the aortic valves of animals treated with warfarin as compared to controls ($p=0.025$) (Figure 1). Animals treated with warfarin showed also significantly higher aortic valve calcification as compared to mice treated with rivaroxaban ($p= 0.005$). No difference was found in the degree of valvular calcification between the animals treated with rivaroxaban and the control group ($p=0.35$).

The effect of the two treatments on atherogenesis was estimated through histological analysis of the innominate arteries. In particular, seriate cryosections of the artery were used to estimate the degree of atherosclerosis and the amount of intimal calcification. The analysis showed that the development of atherosclerosis was similar in the three groups, with no difference in the lesion area between warfarin, rivaroxaban and controls (p= 0.15). Moreover, differently from what seen for the valves, the treatment with warfarin did not induce in our model a significant increase in the accumulation of calcium within the atherosclerotic lesions. In fact, a similar degree of intimal calcification was found in the three groups of mice (Figure 2) (p= 0.53). We also investigated the effect of the treatments on osteogenic conversion of aortic vascular cells. To this purpose we measured ALP activity (a marker of osteoblast-like differentiation) in the aorta of the mice and we found no significant difference between the three groups (see supplemental figure).

In vitro study

The in vitro experiments were performed by using a specific clonal subpopulation of aortic bovine interstitial valve cells (BVIC) that has been previously isolated and characterized by our group.(4, 6) In particular, we know that these cells, in response to different inflammatory stimuli (such as LPS), increase the expression of osteogenic markers and acquire a pro-calcific profile. We performed some experiments to investigate whether addition of rivaroxaban was able to modulate the osteogenic differentiation of valve cells and/or reduce their inflammatory activation. To this purpose, the cells were treated for 12 days with LPS alone (500 ng/ml), LPS+ rivaroxaban (30 µg/ml) or control and at the end of the treatment period we collected cellular mRNA to estimate the expression of ALP (as marker of osteogenic differentiation) and COX-2 (as marker of inflammatory activation). In particular we observed that the treatment with rivaroxaban did not prevent the increase in ALP expression induced by LPS (Figure 3). Viceversa, the administration of Rivaraxoban was able to significantly reduce the over-expression of COX-2 promoted by endotoxin. These

findings suggest that treatment with rivaroxaban does not interfere with the osteogenic differentiation of VIC but can have some protective effect on their inflammatory activation.

Discussion

In the present study we showed that, compared to warfarin, the treatment with rivaroxaban is not associated with an increase accumulation of calcium within the valve leaflets of atherosclerotic mice. In particular, mice treated with rivaroxaban showed a degree of valve calcification similar to control mice and significantly inferior to warfarin (Figure 1). The detrimental effect of warfarin on vascular calcification has been previously described in different animal models and human population studies.(9, 10) However, it was unknown whether the new direct oral anticoagulants, and in particular the blockers of Factor Xa, can have protective or deleterious effects on vascular/valvular calcification. Our in vivo findings indicate that the treatment with rivaroxaban have neutral effects on valve disease progression as compared to control, but showed a significant reduction in the progression of calcific valve degeneration as compared to treatment with warfarin. The latter is known to reduce the vascular accumulation of the c-MGP that is able to prevent the osteogenic conversion of vascular cells (through blockade of BMP-2) and calcium deposition.(11) Our data indicate that, differently from warfarin, rivaroxaban is not exerting such negative modulation of MGP metabolism and do not interfere with the protective mechanisms of the vascular tissue. Nevertheless, rivaroxaban did not show a protective effect on valve disease progression, as the level of calcification was similar between the animal treated with rivaroxaban and controls. The lack of effect of rivaroxaban on osteogenic differentiation of the valve cells was confirmed by the results of in vitro experiments, where the treatment with rivaroxaban did not prevent the osteogenic conversion of VIC induced by inflammatory mediators. In addition both rivaroxaban and warfarin did not modify the osteogenic conversion of arterial aortic cells. Nevertheless, the in vitro studies also showed that rivaroxaban can exert some preventive effect on the inflammatory activation of valve cells, as demonstrated by the significant reduction of COX-2 expression in VIC treated with LPS

(Figure 2). Our data are in line with previous evidence collected in VSMC showing that rivaroxaban can reduce the gene expression of inflammatory molecules in vascular cells.(17) The mechanisms explaining these protective effects are still unclear and might involve the blockade of protease-activated receptor (PAR), a cellular receptor of FXa that can drive the cell activation.(22) However, the in vivo relevance of this effect is unclear, and our data from the animal study seem to suggest a minor importance on valve disease progression. Nevertheless, the inflammatory activation of VIC could be mainly implicated in the early phases of the valve disease, and only investigations performed at earlier stages could clarify whether treatment with rivaroxaban exerts a protective effect on the initiation of valve degeneration.

When we looked at the effects of the two treatments on atherogenesis we found that both rivaroxaban and warfarin did not impact on both atherosclerosis progression and intimal calcification. Data about the potential anti-atherogenic effects of rivaroxaban have been so far controversial. In line with our findings other studies showed that rivaroxaban was ineffective in reducing the development of atherosclerosis, although the pharmacological treatment was followed by an increase in the features of plaque stability. (17) Other investigations showed that rivaroxaban significantly reduced the development of atherosclerosis in mice.(18) Difference between these studies could be explained by the different mouse models and strategies used to quantify atherosclerosis development. However, none of these studies specifically investigated the effect of rivaroxaban on calcium deposition, the main topic of our research. Of note, differently from the aortic valve, the treatment with warfarin did not significantly increase the calcium deposition within the atherosclerotic lesions. This finding is different from previous report showing higher degree of intimal calcification in atherosclerotic mice treated with warfarin (23). Even in this case discrepancy could be due to the different experimental approaches used to quantify calcification and atherosclerosis development. For the present study we quantified atherosclerosis in a single vessel (the innominate artery), by using an experimental approach that is recognized as a reliable and standardized.(24) Moreover, calcification occurring within the innominate artery of ApoE-/- mice is

mainly driven by osteo/chondrogenic conversion of intimale cells,(5) and we know that the lost of c-MGP generation has relevant implication in the development of medial calcification, (25) a phenomenon that has not been quantify in our analysis.

In conclusion, our study showed that warfarin induces calcific valve degeneration in a mouse model of atherosclerosis, without affecting atherosclerosis progression. On the contrary the treatment with rivaroxaban is not accompanied by increase in valve calcification and can exert some anti-inflammatory effects on VIC activation. Overall, our data demonstrated a safer profile of rivaroxaban on the risk of cardiovascular disease progression.

Conflict of interest statement

All authors have no conflicts of interest.

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Author Contributions

Marcello Rattazzi, and Paolo Pauletto conceived the study and wrote the manuscript; Massimo Puato, performed analysis of the data; Mario Plebani performed biochemical analyses; Elisabetta Faggin and Francesco Cinetto, performed histological analyses; Elisa Bertacco and Chiara Nardin performed in vitro investigations and gene expression analyses; Diego Guidolin developed the image analysis tool.

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Figures

Figure 1. Warfarin promotes aortic valve calcification in ApoE -/- mice.

ApoE-/- mice were treated for 8 weeks with warfarin (n=14), rivaroxaban (n=14) or control (n=14). Von Kossa staining was used to quantify the degree of calcium deposition within the aortic valve leaflets. The analysis showed a significant increase in the percentage of valve area occupied by calcium deposits in the mice treated with warfarin as compared to WTD alone (*p= 0.025) or rivaroxaban (**p=0.005). No significant differences were found between rivaroxaban and WTD (p=0.35). The lower panel shows representative images of the aortic sinus in the three treatments (magnification 50x).

Figure 2. Warfarin and Rivaroxaban have similar effects on atherogenesis and intimal calcification in ApoE -/- mice.

ApoE-/- mice were treated for 8 weeks with warfarin (n=14), rivaroxaban (n=14) or control (n=14). Von Kossa staining was used to quantify the atherosclerotic lesion size and intimal calcification in the three treatments. The analysis showed a similar development of both atherosclerosis and intimal calcification in the three groups. The lower panel shows representative images of the innominate artery in the three treatments (magnification 100x).

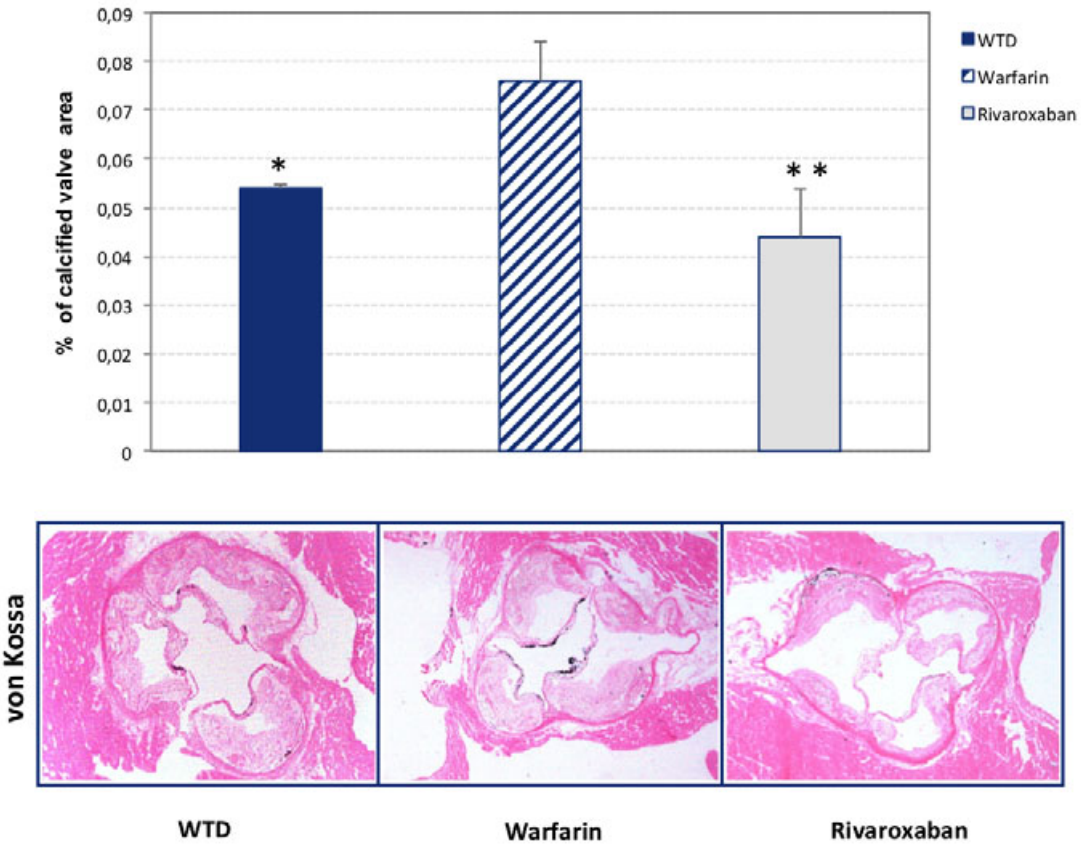
Figure 3. Rivaroxaban reduces inflammatory activation of interstitial valve cells without preventing their osteogenic conversion.

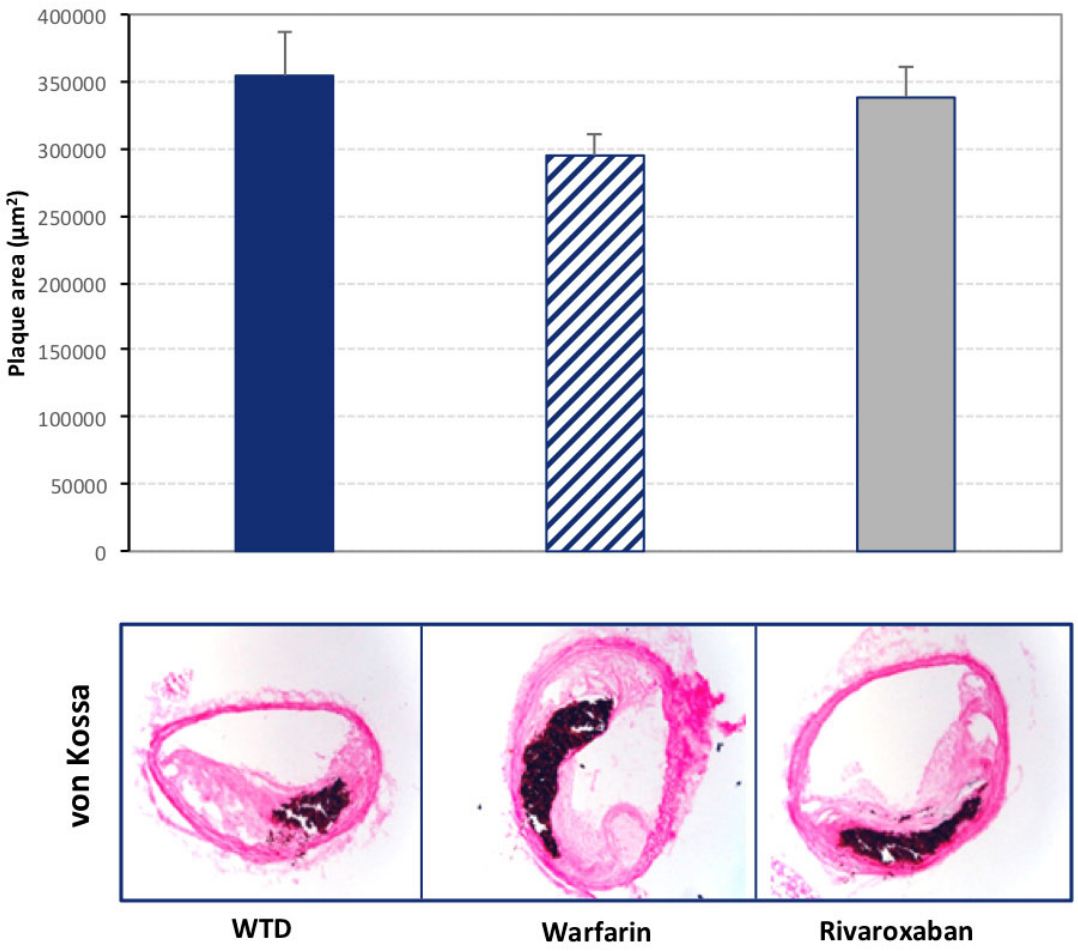
A specific subpopulation of bovine VIC known to acquire a pro-calcific profile in response to treatment with endotoxin was used to test the effect of rivaroxaban on osteogenic and inflammatory activation of valve cells. VIC were treated with LPS (500 ng/ml) with or without addition of rivaroxaban (30 µg/ml) for 12 days. At the end of the treatment mRNA was collected to estimate the gene expression of ALP and COX-2. The analysis showed that rivaroxaban did not

prevent the ALP induction observed in VIC treated with LPS (Panel A), but significantly reduced the over-expression of COX-2 (Panel B).

*p<0.05 vs control, §p<0.05 vs LPS.

Supplemental figure. Warfarin and Rivaroxaban did not modify the ALP activity within the aorta of the ApoE -/- mice. ALP activity was measured in the aorta of the mice to estimate the effect of the treatments on osteogenic conversion of vascular cells. In particular, snap frozen aortas were solubilized with 1% Triton in NaCl 0.9% and supernatants were collected to determine ALP activity and protein content. ALP was determined by using an enzyme activity assay (Chema Diagnostica), and data were normalized to protein content. No differences were found between the three conditions in term of ALP activity within the aorta (ANOVA p=0.54)





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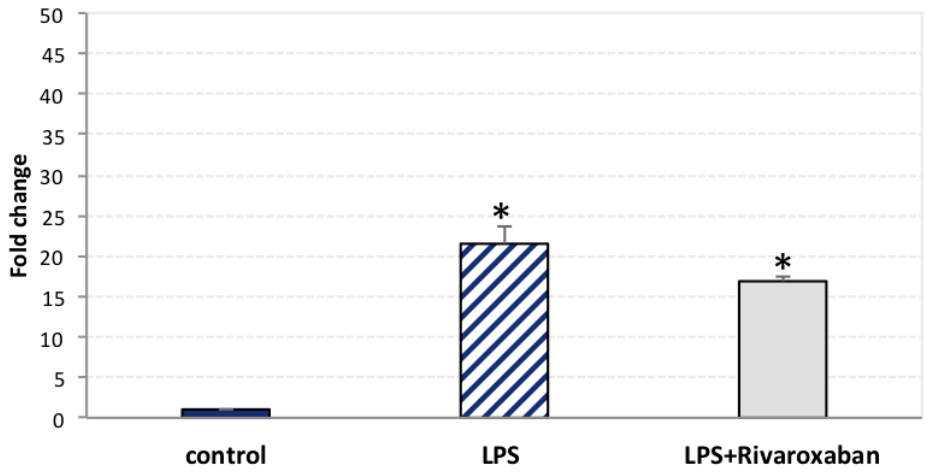

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