Proteomic Analysis of Extracellular Vesicle Cargoes Mirror the Cardioprotective Effects of Rivaroxaban in Patients with Venous Thromboembolism

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Abstract

Venous Thromboembolism (VTE) remains a significant cause of morbidity and mortality worldwide. Rivaroxaban, a direct oral factor Xa inhibitor, mediates anti-inflammatory and cardiovascular-protective effects besides its well-established anticoagulant properties; yet, these remain poorly characterized. Extracellular vesicles (EVs) are considered proinflammatory messengers regulating a myriad of (patho)physiological processes and may be highly relevant to the pathophysiology of VTE. The effects of Rivaroxaban on circulating EVs in VTE patients remain unknown. We have established that differential EV biosignatures are found in patients with non-valvular atrial fibrillation anticoagulated with Rivaroxaban versus warfarin. Here, we investigated whether differential proteomic profiles of circulating EVs could also be found in patients with VTE. We performed comparative label-free quantitative proteomic profiling of enriched plasma EVs from VTE patients anticoagulated with either Rivaroxaban or warfarin using a tandem mass spectrometry approach. Of the 181 quantified proteins, 6 were found to be either exclusive to, or enriched in, Rivaroxaban-treated patients. Intriguingly, these proteins form a cluster tightly involved in negative feedback regulation of inflammatory and coagulation pathways, suggesting that EV proteomic signatures may reflect both Rivaroxaban's anti-coagulatory and anti-inflammatory potential. These findings may be of translational relevance towards characterizing the emerging anti-inflammatory and cardioprotective mechanisms associated with this therapy.

Dataset Brief

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List of abbreviations:

DOAC - direct oral anticoagulant; DVT – deep vein thrombosis; EV - extracellular vesicle; FXa -Factor Xa; MS - mass spectrometry; LC-MS/MS - liquid chromatography tandem mass spectrometry; LFQ - label-free quantification; PAR – protease activated receptor; PE- pulmonary embolism; VKA - vitamin K antagonist, VTE – venous thromboembolism

Keywords

anticoagulation, extracellular vesicles, inflammation, rivaroxaban, venous thromboembolism

Potential clinical relevance

The use of extracellular vesicles as liquid biopsies is emerging. We have used label-free quantification proteomic characterisation to establish that patients with single-episode VTE anticoagulated with Rivaroxaban compared with warfarin demonstrate altered circulating EV profiles with distinct proteomic signatures. The observed differences may indicate a pharmacologic reduction in the pro-thrombotic state in combination with more stable coagulation in Rivaroxaban-treated patients relative to warfarin. These results may be of translational relevance towards characterising the emerging anti-inflammatory and cardiovascular-protective characteristics associated with Rivaroxaban therapy relative to warfarin.

Abstract

Venous Thromboembolism (VTE) remains a significant cause of morbidity and mortality worldwide. Rivaroxaban, a direct oral factor Xa inhibitor, mediates anti-inflammatory and cardiovascular-protective effects besides its well-established anticoagulant properties; yet, these remain poorly characterized. Extracellular vesicles (EVs) are considered proinflammatory messengers regulating a myriad of (patho)physiological processes and may be highly relevant to the pathophysiology of VTE. The effects of Rivaroxaban on circulating EVs in VTE patients remain unknown. We have established that differential EV biosignatures are found in patients with non-valvular atrial fibrillation anticoagulated with Rivaroxaban versus warfarin. Here, we investigated whether differential proteomic profiles of circulating EVs could also be found in patients with VTE.

We performed comparative label-free quantitative proteomic profiling of enriched plasma EVs from VTE patients anticoagulated with either Rivaroxaban or warfarin using a tandem mass spectrometry approach. Of the 181 quantified proteins, 6 were found to be either exclusive to, or enriched in, Rivaroxaban-treated patients. Intriguingly, these proteins form a cluster tightly involved in negative feedback regulation of inflammatory and coagulation pathways, suggesting that EV proteomic signatures may reflect both Rivaroxaban's anti-coagulatory and anti-inflammatory potential. These findings may be of translational relevance towards characterizing the emerging anti-inflammatory and cardioprotective mechanisms associated with this therapy.

Venous thromboembolism (VTE), comprising deep vein thrombosis (DVT) and pulmonary embolism (PE), affects nearly 10 million people worldwide each year, with an estimated fatality rate of nearly 10%. [1] Numerous risk factors are associated with increased risk of developing VTE such as increasing age and obesity. [2], [3] Intriguingly, VTE patients present with a systemic pro-inflammatory state, which potentiates the risk

of recurrence. [4] systemic anticoagulation is the standard of care, both to treat acute VTE and to reduce the risk of recurrence. Historically, vitamin K antagonists such as warfarin or parenteral anticoagulation were the first in line treatment of choice. In the last decade, however, direct oral anticoagulants (DOACs) have emerged and are preferred due to their more predictable pharmacokinetics, fixed dosing and decreased interactions with food and co-prescribed medications. [5], [6] The direct factor Xa inhibitor Rivaroxaban, has been found to be as effective/superior to vitamin K antagonists in preventing recurrent VTE, with similar to reduced rates of clinically significant bleeding events. [7]–[9] Intriguingly, Rivaroxaban also demonstrates anti-inflammatory properties beyond its anticoagulant effects; to date this is primarily attributed to its inhibitory effects on protease-activated receptors (PARs). [10]–[13]

Extracellular vesicles (EVs) are mediators of intercellular communication, regulating a plethora of biological processes via transfer of bioactive molecules such as proteins, lipids and miRNAs [14]. EVs are highly implicated in pro-inflammatory diseases [15]–[19] and can further augment inflammatory signalling by, for example, activating the complement system or shuttling cytokines. [15], [20], [21] EVs can also exacerbate endothelial dysfunction, by inducing the expression of adhesion molecules and inflammatory cytokines, collectively facilitating leukocyte recruitment to the endothelium. [22]–[24] Importantly, the underlying pro-inflammatory phenotype of VTE patients also manifests in increased levels of circulating EVs [25]–[31] and levels of plasma EVs have been implicated as diagnostic or prognostic biomarkers for VTE. [32], [33] Despite the recognised importance of EVs in VTE, no proteomic studies investigating differential EV cargoes have been performed to date. The effects of anticoagulation with Rivaroxaban compared to warfarin on proteomic signatures of circulating EVs following acute VTE and during secondary prevention are currently unknown.

Given this well-established pro-inflammatory state of VTE patients, reflected by heightened levels of circulating EVs together with the systemic anti-inflammatory properties reported for Rivaroxaban, we hypothesised that Rivaroxaban's anti-inflammatory properties may be reflected in the proteomic profiles of circulating EVs. Here, we used LFQ-proteomic profiling to compare the protein content of circulating EVs in patients with single episode VTE anticoagulated with either Rivaroxaban (n=6) or sex-, age-, and BMI-matched warfarin controls (n=6).

We used LFQ-proteomic profiling to assess differences in the vesicular proteome from VTE patients treated with Rivaroxaban and warfarin. Plasma from 6 sex-, age- and BMI-matched individual biological donors for each treatment were enriched for EVs by sucrose cushion ultracentrifugation at an average 120,000 xg and 4 °C for 6 hours. 2 individual donors per treatment cohort were randomly chosen to validate successful EV enrichment by immunoblotting. 20 μg protein from these enriched EV fractions were lysed and resolved on a 10% SDS gel. The presence of transmembrane and soluble EV-associated proteins was investigated according to the guidelines of the International Society of Extracellular Vesicles (ISEV). [36] For a detailed description of the methods, please refer to the online data supplement. Identification of tetraspanins CD63 and CD81 as well as the soluble protein HSP70 confirmed successful enrichment of vesicles (Figure 1A). Detection of albumin indicated co-isolation of plasma proteins (Figure 1A), although study by Tóth *et al.* recently postulated that albumin could be part of the newly established EV protein corona. [37] GO cellular compartment analysis of all identified proteins using FunRich furthermore revealed a highly significant association with the term "exosomes" (p =1.74x10^{-27}; Supplementary Table 1), further substantiating successful enrichment of circulating EVs.

Vesicle preparations were lysed, proteins precipitated, sequentially digested with Lys-C and trypsin and analysed in technical duplicate in a QExactive mass spectrometer. The raw data was searched against a human FASTA using MaxQuant. For the search, a minimum of two peptides per protein needed to be identified, minimal peptide length was set to seven amino acids and a maximum of two miscleavages were allowed. The false discovery rate for peptide and protein identifications in the initial search was set to 0.01. Data filtering included removal of proteins of the reverse data base, proteins only identified by site and common contaminants. For statistical analysis of differential protein expression, data was further filtered to only include proteins that were identified in at least 50% of the patients in at least one treatment cohort. Adopting this approach, 181 proteins were robustly identified across at least 50% of patients in at least one

treatment group (Supplementary Table 2). Pearson correlation analysis of the protein expression revealed robust correlation of the protein LFQ intensities between the patients, averaging at 0.922 ± 0.036 and 0.948 ± 0.023 for Rivaroxaban- and warfarin-treated patients, respectively (Supplementary Table 3), suggesting low inter-donor variability in the protein expression levels within our EV preparations.

Within the 181 identified proteins, one protein, vitamin K-dependent protein Z (PROZ), was found exclusive to the Rivaroxaban-treated cohort (Figure 1B; Table 2), an intriguing finding as low plasma levels of PROZ have been associated with an increased risk of coronary and peripheral artery disease. [38]–[41] Statistical analysis of the expression level of the other shared 180 proteins using a student's*t*- test with a false discovery rate of 5% and a minimal fold change (S_0) of 0.1 (indicated by the black hyperbolic lines in Figure 1C) revealed differential protein quantification for 5 proteins (Table 2). The expression level of the residual 180 proteins remained statistically unchanged (Figure 1C, grey). The functional activity of coagulation factors II (F2), X (F10), Protein S (PROS1) and Protein Z (PROZ) requires vitamin K-dependent post-translational γ-carboxylation, which is inhibited by warfarin. [42] In line with the known mechanisms associated with warfarin therapy [43], proteins upregulated in EVs from Rivaroxaban-treated patients were significantly annotated to the GO biological pathway terms "Gamma-carboxylation of protein precursors" (p =1.62x10^{-8}), "Gamma-carboxylation, transport, and amino-terminal cleavage of proteins" (p =6.31x10^{-8}), and "Post-translational modification: gamma carboxylation and hypusine formation" (p =8.51x10^{-4}; Supplementary Table 1).

4 of the 5 proteins significantly upregulated in the Rivaroxaban cohort (Figure 1C, red; Table 2), namely F2, F10, SERPINA10 and PROS1, are involved in the regulation of coagulation. Warfarin as a vitamin K antagonist inhibits the posttranslational modification of vitamin K-dependent proteins such as F2, F10, PROS1 and PROZ. Although this predominantly affects the functional activity of these proteins [43], it might not be surprising that these proteins indicate higher expression in Rivaroxaban-treated patients. SERPINA10 levels, however, are not vitamin K-dependent. Strikingly, PROZ and SERPINA10 deficiencies have been linked with an increased risk of developing venous thrombosis, making our clinical proteomic findings incredibly relevant. Although some human studies did not find a link, augmented arterial and venous thrombosis was found in both PROZ- as well as SERPINA10-deficient mice. [44]–[46] We further performed STRING network analysis. It revealed that 5 of our 6 proteins found exclusive to or increased in Rivaroxaban patients (F2, F10, PROS1, PROZ, and SERPINA10) formed a distinct network collectively involved in blood coagulation (Figure 1D, red, p =7.11x10^{-5}). Physiologically, SERPINA10 alone is a potent inhibitor of FXIa [47] and complex formation of SERPINA10 with its cofactor PROZ additionally initiates FXa inhibition. [48] Our proteomic changes may therefore reflect the favourable bleeding and anti-thrombotic mechanisms of rivaroxaban reported in clinical trials. [7]–[9]

Besides their anticoagulant effects, PROS1, SEPRINA10 and PROZ can also potentiate anti-inflammatory signalling. For instance, SERPINA10 was recently identified to function as an acute phase protein [49] and subsequently shown to downregulate the expression of pro-inflammatory cytokines (TNFα, IL-1β, IL-6 and CCL3) independent of its anticoagulant effects [50], however, the underlying mechanism remain to be characterised. In line with these findings, low plasma levels of PROZ correlated with increased levels of inflammatory cytokines (CRP and IL-6) in a mouse model of sepsis [51], and in patients with acute myocardial infarction and rheumatoid arthritis (RA) [52], [53], respectively, potentially indicating an anti-inflammatory role for PROZ. Furthermore, PROS1 mediated anti-inflammatory signalling via TAM (Tyro3, Axl, Mer) receptors, negative regulators of inflammation. [54] PROS1 deficiency, on the other hand, increased lung inflammation in a rodent model of lung cancer, which was rescued by addition of exogenous PROS1 in an NF-ϰB dependent manner. [55] Crucially, Rivaroxaban has repeatedly been shown to inhibit pro-inflammatory signalling. While its inhibitory effects have frequently been attributed to the direct inhibition of FXa and downstream PAR signalling [56]–[60], several studies indicated PAR-independent inhibition of NF-ϰB activation [61], [62] and other pro-inflammatory signalling pathways. [63]–[65] Collectively, our observed increases in PROS1, SERPINA10 and PROZ levels in Rivaroxaban-treated patients may indicate a pharmacologically ameliorated underlying pro-inflammatory state in VTE patients.

In conclusion, we have used label-free quantification proteomic characterisation to establish that patients with single-episode VTE anticoagulated with Rivaroxaban compared with warfarin demonstrate altered circulating EV profiles with distinct proteomic signatures. The observed differences may indicate a pharmacologic reduction in the pro-thrombotic state in combination with more stable coagulation in Rivaroxaban-treated patients relative to warfarin. These results may be of translational relevance towards characterising the emerging anti-inflammatory and cardiovascular-protective characteristics associated with Rivaroxaban therapy relative to warfarin. [7]–[9]

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Conflict of Interest

The authors have declared no conflict of interest.

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Tables and Figures

Figure 1: Rivaroxaban modulates a tightly regulated cluster of proteins collectively involved in the regulation of coagulation. Platelet poor plasma samples from individual Rivaroxaban-treated (n=6) and warfarin-treated patients (n=6) were enriched for vesicular fractions by ultracentrifugation. (A) 20 µg protein was separated on a 10% SDS-polyacrylamide gel. Immunoblotting for transmembrane (CD63 (1:1000) and CD81 (1:1000)) and soluble (HSP70 (1:1000)) EV markers confirmed the presence of vesicles in the enrichments across two randomly chosen donors in each cohort. Detection of albumin (1:1000) indicated co-isolation of plasma proteins. (B) Proteomic analysis in technical duplicate revealed a total of 181 proteins identified in at least 50% of at least treatment group, with one protein (PROZ - Vitamin K-dependent protein Z) exclusive in the Rivaroxaban cohort. (C) Volcano plot comparing the expression level of the 180 shared proteins between the Rivaroxaban versus warfarin vesicular proteomes (x-axis, Welch's test difference representing the difference between the mean log2 LFQ values of Rivaroxaban to warfarin EV proteomes; y-axis, the negative log transformed p-value), representing the proteins significantly altered between the two cohorts. The black hyperbolic line indicated the threshold for statistical significance using an FDR of 0.05 and S_0 of 0.1. Of the 180 proteins shared between the cohorts the expression of 5 proteins was significantly increased in the Rivaroxaban cohort (red). The expression level of the remaining 175 proteins remained unaltered (grey). (D) STRING functional protein association network analysis (version 11.5) of the differentially expressed and unique proteins revealed a tight protein-protein interaction network attributed to blood coagulation (red,p =1.43x10^{-7}).

Table 1: Baseline characteristics of study participants.

Characteristics	Rivaroxaban (n = 6)	Warfarin (n = 6)	p-value
Age (years), mean ± SD	70.0 ± 17.2	71.8 ± 18.4	0.862
Sex, n (%) Male Female	2 (33) 4 (67)	2 (33) 4 (67)	1
BMI (kg/m^2)	25.3 ± 6.0	25.9 ± 5.0	0.866
Smoking, n (%) Yes No	2 (33) 4 (67)	0 (0) 6 (100)	0.455
INR, mean ± SD	N/A	2.53 ± 0.27	N/A
TTR (%), mean ± SD	N/A	87.00 ± 10.16	N/A
Time on treatment (months), mean	12.7 ± 7.7	N/A	N/A

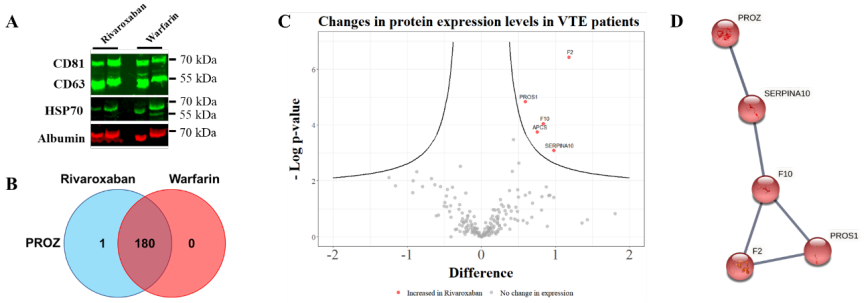
Characteristics	Rivaroxaban (n = 6)	Warfarin (n = 6)	*p*-value
Comorbidities, n (%) Stroke/TIA Ischaemic heart disease Diabetes Hypertension High cholesterol Venous Thromboembolism Chronic kidney disease Chronic liver disease	0 (0) 1 (17) 1 (17) 3 (50) 3 (50) 6 (100) 1 (17) 1 (17)	0 (0) 1 (17) 0 (0) 2 (33) 0 (0) 6 (100) 0 (0) 1 (17)	1 1 1 1 0.182 1 1 1
Medication, n (%) Aspirin Statins Ca^{2+}-channel blocker β-blocker Anti-psychotic	0 (0) 3 (50) 1 (17) 1 (17) 1 (17)	0 (0) 0 (0) 0 (0) 1 (17) 1 (17)	1 0.182 1 1 1

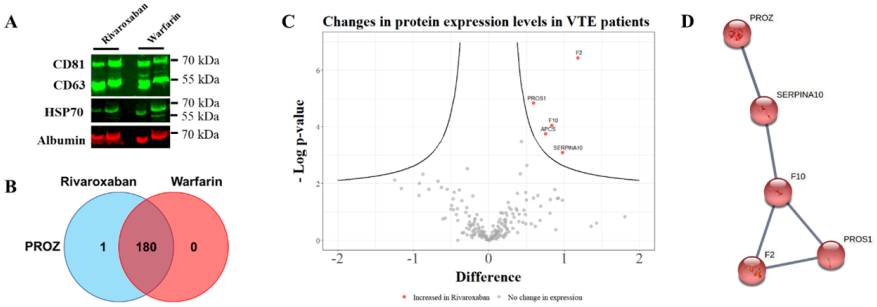
SD – standard deviation, BMI – body mass index, INR – international normalised ratio, TTR – time in therapeutic range, TIA – transient ischemic attack; continuous variables were assessed for statistical difference using a student's t -test, categorical variables were assessed using a Fisher exact test, p -values $<$ 0.05 were regarded statistically significant

Table 2: EV proteins differentially expressed between baseline and 6 months follow-up. One protein was uniquely attributed to the Rivaroxaban-treated patient cohort. Statistical analysis using an unpaired Welch's-test with a false discovery rate of 5 % and a minimal fold change of 0.1 revealed statistically significant increased expression of 5 proteins in EV preparations from Rivaroxaban-treated patients relative to warfarin. Mean label free quantification (LFQ) values for each cohort and the difference of the means are displayed for each protein.

Gene name	Protein name	Rivaroxaban $\log_2$
Proteins unique to Rivaroxaban	Proteins unique to Rivaroxaban	Proteins unique t
PROZ	Vitamin K-dependent protein Z	25.211
Proteins with increased expression in Rivaroxaban	Proteins with increased expression in Rivaroxaban	Proteins with incr
F2	Prothrombin	31.112
SERPINA10	Protein Z-dependent protease inhibitor	25.576
APCS	Serum amyloid P-component	30.324
F10	Coagulation factor X	26.873
PROS1	Vitamin K-dependent protein S	28.831

Figure 1





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