Nonvalvular atrial fibrillation patients anticoagulated with rivaroxaban compared with warfarin exhibit reduced circulating extracellular vesicles with attenuated pro-inflammatory protein signatures

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Abstract

Background: Rivaroxaban, a direct oral factor Xa inhibitor, mediates anti-inflammatory and cardiovascular-protective effects besides its well-established anticoagulant properties; however, these remain poorly characterized. Extracellular vesicles (EVs) are important circulating messengers regulating a myriad of biological and pathological processes and may be highly relevant to the pathophysiology of atrial fibrillation as they reflect alterations in platelet and endothelial biology. However, the effects of rivaroxaban on circulating pro-inflammatory EVs remain unknown.

Objectives: We hypothesized that rivaroxaban’s anti-inflammatory properties are reflected upon differential molecular profiles of circulating EVs.
Atrial fibrillation (AF), the most common sustained cardiac arrhythmia, is associated with a hypercoagulable state, resulting in an increased risk of developing ischemic stroke and systemic embolism. Intriguingly, patients with AF have a characteristic pro-inflammatory state that not only results in endothelial dysfunction, but also directly promotes thrombogenesis. Direct oral anticoagulants are at least as effective as warfarin in preventing stroke and systemic embolism in patients with nonvalvular atrial fibrillation (NVAF), do not require coagulation monitoring while presenting more predictable pharmacokinetics and, importantly, are associated with a more favorable bleeding profile. The direct oral anticoagulant rivaroxaban is a direct inhibitor of activated factor X (FXa), a serine protease that converts prothrombin to thrombin. Both FXa and thrombin mediate pro-inflammatory signaling via protease-activated receptors (PARs), which in turn leads to further activation of coagulation. Due to its inhibitory effects on coagulation and PARs on platelets, low-dose rivaroxaban in combination with aspirin provides dual pathway inhibition, which is postulated to mediate the superior cardiovascular outcomes compared with aspirin alone observed in the COMPASS trial. Hence, long-term FXa inhibition may confer additional benefits mediated by inhibition of proinflammatory signaling; however, underlying molecular mechanisms remain insufficiently characterized.

Methods: Differences in circulating EV profiles were assessed using a combination of single vesicle analysis by Nanoparticle Tracking Analysis and flow cytometry, and proteomics. Results: We demonstrate, for the first time, that rivaroxaban-treated non-valvular atrial fibrillation (NVAF) patients (n=8) exhibit attenuated inflammation compared with matched warfarin controls (n=15). Circulating EV profiles were fundamentally altered. Moreover, quantitative proteomic analysis of enriched plasma EVs from six pooled biological donors per treatment group revealed a profound decrease in highly pro-inflammatory protein expression and complement factors, together with increased expression of negative regulators of inflammatory pathways. Crucially, a reduction in circulating levels of soluble P-selectin was observed in rivaroxaban-treated patients (compared with warfarin controls), which negatively correlated with the patient’s time on treatment.

Conclusion: Collectively, these data demonstrate that NVAF patients anticoagulated with rivaroxaban (compared with warfarin) exhibit both a reduced pro-inflammatory state and evidence of reduced endothelial activation. These findings are of translational relevance toward characterizing the anti-inflammatory and cardiovascular-protective mechanisms associated with rivaroxaban therapy.

KEYWORDS atrial fibrillation, extracellular vesicles, proteomics, rivaroxaban, translational

1 | INTRODUCTION

Extracellular vesicles (EVs) are important messengers containing protein and miRNA signals that regulate a wide range of biological and pathological processes. Both inflammatory signaling and complement activation directly promote the generation of pro-inflammatory EVs and EVs from infected macrophages can further amplify pro-inflammatory responses in vitro and in vivo. As well as their importance in many inflammatory diseases, EVs may play a pivotal role in the pathophysiology of AF, as evidenced by the observation that patients with AF exhibit elevated levels of circulating EVs. Crucially, NVAF patients with left atrial thrombi displayed a further increase in levels of circulating EVs compared with NVAF patients without thrombi and EV levels may predict early recurrence of atrial fibrillation after pulmonary vein isolation. The effect, if
any, of FXa inhibition with rivaroxaban compared with warfarin on plasma EV concentrations, size, and proteomic content is currently unknown. Considering (1) the well-established proinflammatory state in AF patients, (2) that EV profiles (known to correlate with a heightened proinflammatory state) are fundamentally altered in AF patients, and (3) the potent systemic anti-inflammatory effect mediated by rivaroxaban, we hypothesized that EV profiles and molecular signatures would be distinct in AF patients treated with rivaroxaban compared with warfarin. To address our hypothesis, we used a combination of single-vesicle analysis by Nanoparticle Tracking Analysis (NTA) and flow cytometry, and proteomics to compare vesicular signatures from NVAF patients anticoagulated with rivaroxaban compared with warfarin.

2 METHODS

2.1 Patient recruitment and blood collection

Patients with NVAF stably anticoagulated with 20 mg rivaroxaban once daily or warfarin (at a target international normalized ratio [INR] of 2.0–3.0) were recruited following informed consent according to the declaration of Helsinki at the Mater Misericordiae University Hospital, Dublin. Patients had commenced therapy no sooner than 3 months previously for stroke and systemic embolism prevention. Ethical approval was granted by the Research and Ethics Committee (1/378/1821) of the Mater Misericordiae University Hospital. Demographic and clinical data were collected including patient age, body mass index, smoking and alcohol history, medical comorbidities, white cell and platelet count, creatinine clearance, liver profile, medications, and detailed indication for anticoagulation. Patients receiving warfarin had a time in therapeutic range of >55% (measured over 4 months) and an INR in target range at time of sampling.

Exclusion criteria included severe renal impairment (creatinine clearance <30 ml/min), significant liver impairment, known proinflammatory conditions (including systemic lupus erythematosus, inflammatory bowel disease, rheumatoid arthritis), active malignancy, previous stroke or systemic inflammation, antiphospholipid syndrome, strong thrombophilia (e.g., antithrombin deficiency), individuals aged <18 years, patients receiving inhibitory CYP3A4 and P-glycoprotein medications or platelet inhibitors, bleeding or platelet function disorders, and thrombocytopenia (platelet count <150 × 10⁹/ml).

Blood was collected via venipuncture with a 20-gauge needle in two 3 ml 3.2% citrate (1:9, citrate:blood) monovettes (Sarstedt) per patient and spun at 1811g for 6 min at room temperature in an Eppendorf 5810 centrifuge (Eppendorf). Supernatants were transferred into a 15 ml canonical tube, leaving at least 5 mm above the buffy coat. Plasma was centrifuged again as indicated previously. Samples were aliquoted to prevent freeze-and-thaw cycles and stored at −80°C until analysis. Plasma platelet counts were checked in a Sysmex XN (Sysmex Corporation) to ensure <10 × 10⁹ platelets/L.

2.2 Nanoparticle tracking analysis

Particle size distribution in plasma was determined by NTA using a NanoSight NS300 system (Malvern Technologies) configured with a 488-nm laser and a high-sensitivity scientific CMOS camera, as described previously. Plasma was diluted (1:250–1:1000) in particle-free phosphate-buffered saline (PBS; Gibco) to achieve an initial number of 10 to 60 particles per frame. Sample analysis was performed at 25°C and a constant flow rate of 50. The 15 × 60 s videos were captured with a camera level of 13 and data were analyzed using NTA 3.1.54 software with a detection threshold of 10 as before.

2.3 Flow cytometry

Flow cytometry analysis of circulating EVs was performed using a CytoFlex LX (Beckman Coulter). The 100-, 300-, 500-, and 900-nm analysis gates were established using Gigamix beads (Biocytex). As polystyrene beads are solid spheres with a refractive index of 1.61, whereas vesicles consist of a core and a shell with refractive indices of 1.38 and 1.48, respectively, compensation for differences in refractive indices was performed using Rosetta calibration beads (Exometry) and the Rosetta calibration software (version 1.12). This calibration is based on the Mie theory and used to relate side scatter intensities (in arbitrary units) to EV diameter (in nm) for a given refractive index. Data analysis was performed using Kaluza (version 2.1, Beckman Coulter). The 30 µL platelet-poor plasma (PPP) was diluted with 520 µL 0.22 µm filtered PBS. Samples were further diluted 1:20 to prevent EV swarming and analyzed in triplicate at a constant flow rate of 10 µL/min for 2 min or until 100 000 events were recorded. A buffer-only (0.22 µm filtered PBS) sample was assayed using the same settings and during the same experiment as the samples and background vesicle counts were subtracted from the respective samples.

The MIFlowCyt-EV checklist has been completed and is attached in the supplementary material (Supplementary Table S1).

2.4 Sucrose cushion ultracentrifugation

PPP was enriched for vesicular contents three times by ultracentrifugation at average 110 000 g (37 000 rpm, k-factor = 137.1) and 4°C for 70 min each on a 30% sucrose/198 mM Tris (pH 7.4) cushion in an Optima MAX ultracentrifuge (Beckman Coulter) with an MLS-50 swing out rotor (Beckman Coulter). After each spin, the vesicular fraction was removed, washed with PBS, and layered on top of a fresh sucrose cushion. The enriched vesicles were collected and resuspended in 2× RIPA lysis buffer (100 mM Tris pH 8.0, 300 mM NaCl, 2% Triton-X 100, 0.2% SDS, 1% sodium deoxycholate) containing protease inhibitors (Roche) and lysed for 30 min on ice before assessment of the protein concentration using a Bradford assay (BioRad).
2.5 | Immuno blotting

A total of 20 µg protein from each sample was precipitated, resuspended in 2x RIPA lysis buffer and mixed with equal volumes of reducing sample buffer (125 mM Tris HCl pH 6.7, 3% SDS, 7 mM dithiothreitol, 20% glycerol, 0.05% bromophenol blue). Proteins were separated on a 10% polyacrylamide gel before protein transfer onto a polyvinylidene fluoride membrane. Membranes were blocked in 5% skim milk for 1 h at room temperature, washed 3 x 10 min with TBS-T (TBS + 0.1% Tween-20) before incubation with primary antibodies at a dilution of 1:1000 unless otherwise indicated overnight at 4°C. CD9 (sc-59140), Annexin 1 (sc-12740), Albumin (sc-51515), and APO A1 (sc-376818) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Dallas). Polyclonal CD63 (#CAB5271) was purchased from AntibodyGenie. Following 3 x 10 min washes in TBS-T, membranes were incubated with horseradish peroxidase-coupled secondary antibodies for 1 h at room temperature. Proteins were detected using enhanced chemiluminescence substrate.

2.6 | Mass spectrometry

A total of 80 µg protein was precipitated with 95% acetone (1:4, sample volume: acetone) overnight at -20°C. Mass spectrometry sample preparation was performed as described previously. Briefly, proteins were resuspended in 8M urea/25 mM Tris-HCl and incubated at 37°C for 1 h. Disulphide bonds were reduced with 5 mM DTT and alkylated with 15 mM iodoacetamide. Proteins were sequentially digested with Lys-C (1:100, Promega) followed by Trypsin (1:100, Promega). Peptides were purified by solid-phase extraction using ZipTip C<sub>18</sub> pipette tips (Millipore), resuspended in 0.5% acetic acid/2.5% acetonitrile, and analyzed in technical sextuplicate using a Q Exactive mass spectrometer (MS) connected to a Dionex UltiMate 3000 RSLCnano chromatography system (Thermo Scientific) as described previously. The samples were loaded into a fused silica emitter (internal diameter 75 µm) packed with Reprocil Pur C18 (1.9 µm, 12 cm in length) reverse-phase media, pulled by a laser puller (P2000, Sutter Instruments), and were separated with an increasing acetonitrile gradient over 60 min at a flow rate of 250 nl/min directly into a Q Exactive MS. The MS was operated in positive ion mode with a capillary voltage of 1500 V, dry gas flow of 3 L/min, and a dry temperature of 180°C. All data were acquired with the instrument operating in trapped ion mobility spectrometry mode. Trapped ions were selected for MS/MS using parallel accumulation serial fragmentation. A scan range of (100–1700 m/z) was performed at a rate of 10 parallel accumulation serial fragmentation MS/MS frames to 1 MS scan with a cycle time of 1.16 s.

Tandem mass spectra were searched against a human FASTA (Uniprot, July 2018) using the freely available MaxQuant proteomics software (version 1.6.2.3). MaxQuant analysis included an initial search with a precursor mass tolerance of 20 ppm, the results of which were used for mass recalibration. In the main Andromeda search precursor, mass and fragment mass had an initial mass tolerance of 6 and 20 ppm, respectively. The search included fixed modification of carbamidomethyl cysteine, Minimal peptide length was set to seven amino acids, and a maximum of two miscleavages was allowed. The false discovery rate (FDR) was set to 0.01 for peptide and protein identifications. MaxQuant assigns label-free quantitative (LFQ) values to each identified protein.

2.7 | ELISA

ELISAs were used to determine platelet activation markers in plasma. Sandwich ELISAs for P-selectin (CD62P, R&D Systems, Minneapolis, MN), platelet factor 4 (PF4, R&D Systems), and thrombospondin 1 (TSP-1 R&D Systems) were performed according to the manufacturer’s instructions. All standards and samples were assayed in triplicate.

2.8 | Statistical analysis

Statistical analysis of the LFQ intensities was performed using Perseus (version 1.6.2.1) or R (version 3.6.1) as before. LFQ values were log2 transformed, protein identifications were filtered to eliminate identifications from the reverse database, proteins only identified by site, and common contaminants. For statistical analysis, only proteins identified in at least 50% of the replicate runs in at least one treatment group were included. Differences in protein expression were determined using an unpaired t-test with an FDR of 5% and a minimal fold change (S<sub>0</sub>) of 0.1 within the Perseus software. Gene ontology (GO) analysis was performed in FunRich (http://www.funrich.org/, version 3.1.3).

Statistical analysis of differences in EV levels and protein expression determined by ELISA were assessed in RStudio (version 1.2.1335). Data were tested for normal distribution using a Shapiro-Wilk Test. Normally distributed data were assessed for statistical significance using an unpaired t-test. Non-normally distributed data were tested using a Mann-Whitney U test. p-values below .05 were regarded statistically significant.
3 | RESULTS

3.1 | Rivaroxaban therapy reduces the levels of circulating small EVs

We evaluated circulating EV concentration and size from NVAF patients stably anticoagulated with rivaroxaban for > 3 months compared with dose-adjusted (INR 2.0–3.0) warfarin controls. Baseline characteristics of participants enrolled in this study are outlined in Table 1. NTA particle distribution profiles of plasma from rivaroxaban-treated patients were characterized by a reduced level of circulating small EVs in the exosomal size range (0–200 nm) when compared to warfarin controls (Figure 1A). Total particle counts were significantly reduced \( (p = .003, \text{Figure 1B}) \), while vesicle mode size remained unchanged (Figure 1C). Flow cytometry analysis of large EVs between 200–1000 nm revealed no difference in either total particle count \( (p = .427, \text{Figure 1D}) \) or vesicle size between treatment groups (Figure 1E). Pearson correlation \( (r) \) analysis revealed a moderate but significant correlation between total particle levels measured by flow cytometry and NTA \( (r = .575, p = .009, \text{Figure 1F}) \).

3.2 | Rivaroxaban modulates pro-inflammatory vesicle proteomic profiles

LFQ-proteomic profiling was used to examine the molecular content of circulating EVs in NVAF patients treated with rivaroxaban in comparison to warfarin. To minimize individual variability and uncover pharmacological differences, PPP from six matched biological donors anticoagulated with either rivaroxaban or warfarin were pooled prior to enrichment for vesicular fractions using sucrose cushion ultracentrifugation. No significant difference in baseline patient characteristics were observed (Table 2).

Enriched vesicular fractions were assessed for the presence of classic transmembrane and soluble EV markers (CD9, CD63, Annexin1, Albumin)\(^{39}\) (Figure 2A). Enriched vesicles were then lysed and proteins precipitated, followed by sequential digestion and analysis in sextuplicate by LC-MS/MS. Adopting this approach, a total of 144 proteins were identified in at least 50% of the replicate runs in at least one treatment group, including 2 proteins suprabasin (SBSN) and immunoglobulin lambda variable 2-14 (IGLV2-14) \( (\text{Figure 2B}) \) both uniquely identified in rivaroxaban-treated patients. See Supplemental Table S2 for detailed information (including log2 transformed LFQ values, peptide sequences and sequence coverage) for these 144 proteins.

Statistical analysis of the 142 overlapping proteins using a student's t-test with a false discovery rate of 0.05 and a minimal fold change \( (S_{0.1}) \) of 0.1, denoted by the black hyperbolic lines (Figure 2C), revealed 100 proteins that were differentially expressed between treatment groups. Of these 100 proteins, 52 were significantly lower in the rivaroxaban-treated compared with the warfarin-treated cohort (Figure 2C, blue dots; Supplementary Table S3), including S100 calcium-binding proteins A7 (S100A7), S100A8 and S100A9. Furthermore, we identified increased expression of 48 vesicular proteins in rivaroxaban-treated patients compared with warfarin-treated patients (Figure 2C, red dots; Supplementary Table S3). Interestingly, amongst these 48 proteins, we identified increased expression of complement 4 binding protein α and β chains (C4BPA/B), which are negative regulators of classical complement activation.\(^{39}\) Moreover, unsupervised hierarchical clustering with Euclidian distances of the all 102 differential proteins (100 differentially expressed and 2 unique proteins) revealed a complete separation of our rivaroxaban and warfarin treated patient cohorts (Figure 2D).

Using an independent in-depth timsTOF mass spectrometry approach, the differential expression of several peptides was further validated in whole plasma from each individual donor. Comparing to statistically significant peptide differences observed in the pooled samples, we validated differential expression for several peptides/proteins including C4BPA (Supplementary Table S4). As this validation was performed in plasma as opposed to enriched EVs (due to limited sample availability), the wide dynamic range unfortunately precluded the identification of low-abundant peptides/proteins, including the S100A calcium binding proteins.

In order to classify functional differences in the vesicular proteome of rivaroxaban compared with warfarin-treated patients, we performed gene ontology (GO) analysis. GO cellular component analysis of all 144 identified proteins revealed significant enrichment for several GO terms, with 65% of the proteins attributed to proteins in rivaroxaban-treated patients additionally (Supplementary Table S5), further highlighting rivaroxaban's inhibitory effect on complement activation. Collectively, these data further underscore the anti-inflammatory effect of rivaroxaban mediated by downregulation of the classical complement pathway relative to warfarin.

3.3 | Rivaroxaban treatment exhibits distinct effects on circulating platelet and endothelial cell markers

Given that on average 29% of our 144 identified proteins overlap with platelet-derived EVs\(^{37,40,41}\) (with marginal overlap with lymphocyte\(^{42}\), erythrocyte\(^{43}\) and endothelial\(^{44}\) EV proteomes; Supplementary Table S6), we investigated whether the reduced levels of circulating EVs may be due to attenuated platelet activation in rivaroxaban compared with warfarin-treated patients. In the rivaroxaban treated cohort we identified significantly lower levels of soluble P-selectin (sP-selectin) \( (p = .0486, \text{Figure 3A}) \), whereas levels of thrombospondin 1 (TSP-1) remained unchanged \( (p = .640, \text{Figure 3B}) \). sP-selectin and TSP-1 can be released from both activated platelets and endothelial cells.\(^{45,46}\) Therefore, we further investigated the cellular origin of sP-selectin in our cohort by assessing circulating levels of the platelet-specific marker, PF4. Compared with sP-selectin, PF4 is a soluble rather than a cleaved membrane protein; however, it is exclusively expressed in platelets. We found no...
## Table 1: Baseline characteristics of study participants

| Characteristics                        | Rivaroxaban (n = 8) | Warfarin (n = 15) | p Value |
|----------------------------------------|---------------------|-------------------|---------|
| Age (y), mean ± SD                     | 69.6 ± 12.5         | 73.3 ± 6.3        | .349    |
| Sex, n (%)                             |                     |                   |         |
| Male                                   | 4 (50.0%)           | 10 (66.6%)        | 1       |
| Female                                 | 4 (50.0%)           | 5 (33.3%)         |         |
| BMI (kg/m²)                            | 30.44 ± 8.56<sup>a</sup> | 27.35 ± 7.08     | .432    |
| Smoking<sup>b</sup>, n (%)             |                     |                   |         |
| Current                                | 0 (0%)              | 2 (13.3%)         | .123    |
| Ex                                     | 3 (50.0%)           | 1 (6.6%)          |         |
| Non                                    | 3 (50.0%)           | 12 (80.0%)        |         |
| INR, mean ± SD                         | N/A                 | 2.51 ± 0.29       |         |
| TTR (%), mean ± SD                     | N/A                 | 77.6 ± 12.5       |         |
| Time on treatment (mo), mean           | 30                  | N/A               |         |
| Comorbidities, n (%)                   |                     |                   |         |
| Stroke/TIA                             | 0 (0%)              | 0 (0%)            | 1       |
| Ischemic heart disease                 | 1 (12.5%)           | 0 (0%)            | .348    |
| Diabetes                               | 1 (12.5%)           | 2 (13.3%)         | 1       |
| Hypertension                           | 6 (75.0%)           | 11 (73.3%)        | 1       |
| High cholesterol                       | 3 (37.5%)           | 9 (60.0%)         | .400    |
| Venous Thromboembolism                 | 0 (0%)              | 0 (0%)            | 1       |
| Chronic kidney disease                 | 0 (0%)              | 0 (0%)            | 1       |
| Chronic liver disease                  | 0 (0%)              | 0 (0%)            | 1       |
| Other                                  |                     |                   |         |
| Nonischemic heart disease              | 1 (12.5%)           | 4 (26.6%)         | .621    |
| Hemochromatosis                        | 0 (0%)              | 1 (6.6%)          | 1       |
| Hypothyroid                            | 1 (12.5%)           | 1 (6.6%)          | 1       |
| Pulmonary disease                      | 1 (12.5%)           | 0 (0%)            | .348    |
| Medication, n (%)                      |                     |                   |         |
| Aspirin                                | 0 (0%)              | 0 (0%)            | 1       |
| Statins                                | 2 (25.0%)           | 9 (60.0%)         | .193    |
| Antihypertensives                      |                     |                   |         |
| ACE inhibitors                         | 2 (25.0%)           | 5 (33.3%)         | 1       |
| ARB                                    | 2 (25.0%)           | 5 (33.3%)         | 1       |
| Diuretic                               | 2 (25.0%)           | 4 (26.6%)         | 1       |
| Ca²⁺-channel blocker                   | 1 (12.5%)           | 4 (26.6%)         | .621    |
| Other                                  |                     |                   |         |
| β-blocker                              | 7 (87.5%)           | 9 (60.0%)         | .345    |
| Antiarrhythmic                         | 3 (37.5%)           | 3 (20.0%)         | .621    |
| Antipsychotic                          | 1 (12.5%)           | 0 (0%)            | .348    |
| Antiepileptic                          | 1 (12.5%)           | 0 (0%)            | .348    |
| Antihyperglycemic                      | 1 (12.5%)           | 1 (6.6%)          | 1       |

Abbreviations: ACE, angiotensin-converting-enzyme; ARB, angiotensin receptor blocker; BMI, body mass index; INR, international normalized ratio; N/A, not available; SD, standard deviation; TIA, transient ischemic attack; TTR, time in therapeutic range.

<sup>a</sup>Missing data on three patients.

<sup>b</sup>Missing data on two rivaroxaban-treated patients.
differences between the cohorts ($p = .680$, Figure 3C). However, Pearson correlation analysis revealed a strong and highly significant positive correlation between PF4 and TSP-1 levels ($r = .859$, $p < .0001$, Figure 3D), whereas PF4 levels correlated only moderately and nonsignificantly with sP-selectin ($r = .675$, $p = .355$, Figure 3E), suggesting that the altered sP-selectin in our cohort may have originated from activated endothelial cells. Because increased levels of sP-selectin have been associated with endothelial dysfunction, these data may point toward clinically important vascular protective effects of rivaroxaban.

Even though not statistically significant because of very small patient numbers in the rivaroxaban cohort, we also observed a negative correlation of all three circulating markers with the patient’s time on treatment (Supplementary Figure S1), potentially suggesting that the rivaroxaban-mediated cardioprotective effects may become more pronounced as time on treatment progresses.

4 | DISCUSSION

Here, we characterized the hitherto unknown impact of anticoagulation with rivaroxaban compared to warfarin on circulating EV size, concentration, and proteomic profiles. This was based on the hypothesis that rivaroxaban-mediated anti-inflammatory properties could modulate the proinflammatory phenotype exhibited by NVAF patients, a phenotype that may be highly relevant to cardiovascular outcomes. Our analysis revealed that rivaroxaban significantly reduces levels of circulating small EVs compared with warfarin controls, suggesting that rivaroxaban attenuates the baseline proinflammatory state exhibited by NVAF patients relative to warfarin.

Through comparative quantitative proteomic analysis of enriched plasma EVs, we found a profound decrease in highly proinflammatory protein expression and complement factors, together with increased expression of negative regulators of inflammatory
pathways. Pooling before proteomic analysis was crucial to highlight therapeutic differences and minimize biological variation, especially in light of the limited results of a previous study comparing nonpooled plasma proteomic differences between rivaroxaban and warfarin treated patients. Our focus on investigating the circulating EV proteome rather than on the total plasma proteome was also

| Characteristics                            | Rivaroxaban (n = 6) | Warfarin (n = 6) | p Value |
|--------------------------------------------|---------------------|-----------------|---------|
| Age (y), mean ± SD                         | 68.5 ± 14.4         | 69.3 ± 4.4      | .895    |
| Sex, n (%)                                 |                     |                 |         |
| Male                                       | 4 (66.6%)           | 4 (66.6%)       | 1       |
| Female                                     | 2 (33.3%)           | 2 (33.3%)       |         |
| BMI (kg/m$^2$)                             | 30.44 ± 8.56        | 27.60 ± 5.82    | .529    |
| Smoking, n (%)                             |                     |                 |         |
| Current                                    | 0                   | 0               | .546    |
| Ex                                         | 3 (50.0%)           | 1 (16.6%)       |         |
| Non                                        | 3 (50.0%)           | 5 (83.3%)       |         |
| INR, mean ± SD                             | N/A                 | 2.63 ± 0.33     | –       |
| TTR (%), mean ± SD                         | N/A                 | 79.0 ± 15.4     | –       |
| Time on treatment (mo), mean               | 30                  | N/A             | –       |
| Comorbidities, n (%)                       |                     |                 |         |
| Stroke/TIA                                 | 0 (0%)              | 0 (0%)          | 1       |
| Ischemic heart disease                     | 1 (16.6%)           | 0 (0%)          | 1       |
| Diabetes                                   | 1 (16.6%)           | 0 (0%)          | 1       |
| Hypertension                               | 5 (83.3%)           | 4 (66.6%)       | 1       |
| High cholesterol                           | 2 (33.3%)           | 4 (66.6%)       | .567    |
| Venous Thromboembolism                     | 0 (0%)              | 0 (0%)          | 1       |
| Chronic kidney disease                     | 0 (0%)              | 0 (0%)          | 1       |
| Chronic liver disease                      | 0 (0%)              | 0 (0%)          | 1       |
| Other                                      |                     |                 |         |
| Nonischemic heart disease                  | 1 (16.6%)           | 3 (50%)         | .546    |
| Haemochromatosis                           | 0 (0%)              | 1 (16.6%)       | 1       |
| Hypothyroid                                | 0 (0%)              | 1 (16.6%)       | 1       |
| Pulmonary disease                          | 1 (16.6%)           | 0 (0%)          | 1       |
| Medication, n (%)                          |                     |                 |         |
| Aspirin                                    | 0 (0%)              | 0 (0%)          | 1       |
| Statins                                    | 1 (16.6%)           | 4 (66.6%)       | .242    |
| Antihypertensives                          |                     |                 |         |
| ACE inhibitors                             | 2 (33.3%)           | 2 (33.3%)       | 1       |
| ARB                                        | 2 (33.3%)           | 2 (33.3%)       | 1       |
| Diuretic                                   | 1 (16.6%)           | 1 (16.6%)       | 1       |
| Ca$^{2+}$-channel blocker                  | 1 (16.6%)           | 2 (33.3%)       | 1       |
| Other                                      |                     |                 |         |
| β-blocker                                  | 5 (83.3%)           | 3 (50.0%)       | .546    |
| Antiarrhythmic                             | 3 (50.0%)           | 2 (33.3%)       | 1       |
| Antipsychotic                              | 1 (16.6%)           | 0 (0%)          | 1       |
| Antiepileptic                              | 1 (16.6%)           | 0 (0%)          | 1       |
| Antihyperglycemic                          | 1 (16.6%)           | 0 (0%)          | 1       |

Abbreviations: ACE, angiotensin-converting-enzyme; ARB, angiotensin receptor blocker; BMI, body mass index; INR, international normalized ratio; N/A, not available; SD, standard deviation; TIA, transient ischemic attack; TTR, time in therapeutic range.
considered critical because previous proteomic characterization of both plasma and circulating EVs from patients with ST-elevation myocardial infarction compared with controls revealed striking differences in the EV proteome that were unmatched in corresponding plasma samples. Intriguingly, these unique EV-associated differential proteins were associated with higher diagnostic accuracy for ST-elevation myocardial infarction when compared with any plasma proteome changes, further underscoring our chosen methodology and highlighting the translational relevance of our investigation.

Several proteins modulating inflammatory pathways, including S100A8, S100A9, and C4BPA/B, were identified to be significantly altered between circulating EVs isolated from rivaroxaban-treated NVAF patients compared with those managed with warfarin. Intriguingly, S100A8 and S100A9 are highly pro-inflammatory proteins that can form a heterodimeric complex and their lower expression predicts contribution to a rivaroxaban-mediated anti-inflammatory phenotype. S100A8 and S100A9 are platelet- and neutrophil-derived proteins that play pivotal roles in inflammation and thrombosis and, importantly, increased plasma levels of S100A8/A9 (also known as myeloid-related protein-8/14 or calprotectin) are associated with an increased risk of myocardial infarction and cardiovascular death. The observation that their expression was reduced in rivaroxaban compared with warfarin-treated patients therefore generates the interesting hypothesis that an additional clinical cardioprotective and mortality-sparing mechanism has been uncovered. Moreover, S100A8/A9 can induce NF-κB signaling, leading to expression of pro-inflammatory cytokines, chemokines, and metastatic factors. Rivaroxaban has repeatedly been shown to inhibit NF-κB signaling, leading to expression of pro-inflammatory cytokines, chemokines, and metastatic factors. Rivaroxaban has repeatedly been shown to inhibit NF-κB signalling. Therefore, the observation that S100A8/A9 expression was lower in the rivaroxaban cohort may represent a mechanism through which rivaroxaban treatment influences NF-κB signaling. Intriguingly, in rivaroxaban-treated patients, these proteins exhibited the largest decrease in expression, again supporting our hypothesis that circulating EV profile signatures are a powerful surrogate marker for rivaroxaban’s anti-inflammatory effects. In keeping with this theme, C4BP was found to be expressed at higher amounts in rivaroxaban-treated patients compared with patients managed on warfarin. C4BP is a negative regulator of both
the classical and lectin pathways of complement activation, which represents an innate mechanism of host defense against pathogens.\textsuperscript{39} C4BP accelerates degradation of the C3-convertase C4bC2a that physiologically induces the release of anaphylatoxin C3a,\textsuperscript{59} reducing pro-inflammatory signaling. Collectively, the observed increased C4BPA/B expression, together with a decrease in C1s, C1r and C1q expression and in combination with GO biological pathway analysis, supports the well-established\textsuperscript{20} biological role played by rivaroxaban (as distinct to warfarin) in specifically attenuating classical complement activation. Collectively, these data suggest that comprehensive characterization of circulating EVs reveals crucial information, that may be of translational relevance towards characterizing the underlying anti-inflammatory mechanisms associated with rivaroxaban therapy.

Previous studies have demonstrated that rivaroxaban ameliorates endothelial dysfunction,\textsuperscript{16,60–62} a mode of action that may be central to its cardioprotective properties. Therefore, we investigated rivaroxaban’s effect on well-known circulating platelet and endothelial activation markers. Our findings revealed a reduction in circulating levels of sP-selectin, whereas TSP-1, and PF4 levels remained unchanged. Although all markers are expressed in platelets, sP-selectin and TSP-1 may also be endothelial-derived,\textsuperscript{55,64} suggesting that rivaroxaban treatment could exhibit distinct effects on platelets and endothelial cells. To further investigate the cellular origin of sP-selectin and TSP-1, we performed Pearson correlation analyses. We observed a highly significant, strong positive correlation of TSP-1 and PF4 levels, whereas PF4 correlated moderately but not significantly with sP-selectin. Because PF4 is exclusively expressed in platelets, these results suggest that the alterations denoted in sP-selectin between our cohorts may be endothelial-derived.

In line with these findings, increased levels of sP-selectin have previously been attributed to endothelial dysfunction under oxidative stress\textsuperscript{48} and hyperglycemic conditions.\textsuperscript{47} Augmented generation of reactive oxygen species represents a major contributor to the development of endothelial dysfunction.\textsuperscript{63} Intriguingly, rivaroxaban ameliorated oxidative stress in vitro and in animal models by down-regulation of NADPH oxidase and NOS2 and upregulation of eNOS via inhibition of FXa/thrombin-induced PAR activation.\textsuperscript{61,64} The role of FXa and thrombin in PAR-mediated activation of endothelial cells is well known.\textsuperscript{65} Endothelial cells express PAR-1, mainly activated by thrombin, as well as PAR-2, cleaved by FXa.\textsuperscript{65} Activation of human umbilical vein endothelial cells with thrombin led to increased expression of pro-inflammatory cytokines (IL-8, CXCL1, CXCL2, MCP-1) and several cellular adhesion molecules (E-Selectin, VCAM-1, ICAM-1) in a PAR-dependent manner, which could be reversed by preincubation with rivaroxaban.\textsuperscript{64,66–68} Similarly, rivaroxaban treatment dampened FXa-induced upregulation of ICAM-1 and MCP-1 in endothelial progenitor cells\textsuperscript{69} and induced neovascularization, cell migration, and enhanced blood flow in diabetic mice after hindlimb ischemia,\textsuperscript{61,64} indicating ameliorated endothelial dysfunction and increased vascular protection. Consistent with the vascular protective properties observed in these experimental models, our findings may,
for the first time, indicate ameliorated endothelial dysfunction in a clinical cohort of NVAF patients anticoagulated with rivaroxaban relative to warfarin. Intriguingly, herein, circulating levels of all markers assessed also correlated negatively with the patient’s time on rivaroxaban treatment, potentially pointing to a clinically important progressive vascular protective effect, which may be of importance toward elucidating the mechanisms underlying the cardioprotective properties of rivaroxaban in clinical trials.16,60

There are some limitations associated with the present study. Because patients with severe renal impairment were excluded, the effects of lower dose rivaroxaban (15 mg/d) in renally impaired patients may not reflect the results obtained. Sample size in our cross-sectional study was also limited; however, we maximized the potential to uncover clinically meaningful results by utilizing highly matched subjects and because of limited sample availability, we pooled samples prior to EV enrichment for proteomic analysis. We did, however, validate the differential expression of several peptides/proteins in whole plasma using an in-depth MS approach. Additionally, although the differences in EV levels and inflammatory protein expression observed in our study underline the pleiotropic effects of rivaroxaban therapy, this study was not designed to determine if a specific clinical outcome is linked to a distinct EV/proteomic profile. To assess the pathological relevance, a large prospective clinical study with a sufficient sample size would be needed to determine if the observed differences link to clinical outcomes. Yet, evidence from the literature suggests that EV levels may predict treatment outcome in cancer,70–72 potentially suggesting a pathological relevance of the observed discrepancies. Last, although in-depth flow cytometry to investigate differences in the origin of the EVs would be hugely informative regarding differential effects of rivaroxaban on platelets and endothelial cells, these analyses were outside the scope of the current study. However, in line with our observations, others have recently seen no difference in the levels of circulating platelet- and endothelial cell-derived large EVs >700 nm in a similar patient cohort with NVAF anticoagulated with rivaroxaban compared to warfarin.73 Although the alterations we saw were in smaller EVs below 200 nm, there are currently unfortunately no validated methods or markers that enable assessment of cellular origin of such vesicles.38

In conclusion, the data herein demonstrate that NVAF patients anticoagulated with rivaroxaban (compared with warfarin) exhibit both a profoundly attenuated pro-inflammatory state and circulating evidence of reduced endothelial activation. Moreover, EV signatures were revealed to be powerful biological “sensors” of rivaroxaban-mediated anti-inflammatory effects, an exciting finding that may be leveraged when planning future studies exploring personalized management strategies, such as identification of those patients most likely to benefit from therapy, particularly in jurisdictions where access to therapy may be restricted. Finally, and crucially, rivaroxaban-mediated vascular protection was revealed, for the first time, to become more profound over time, suggesting that prolonged therapy may be critical in optimal rivaroxaban-mediated cardioprotection.

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CONFLICTS OF INTEREST
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AUTHOR CONTRIBUTIONS
Luisa Weiss, John Keaney, Paulina B. Szklanna, Alfonso Blanco, Barry Kevane, Sean Murphy, Fionnuala Ní Áinle, and Patricia B. Maguire designed research; Luisa Weiss, Paulina B. Szklanna, Tadhg Prendiville, Wido Uhrig, Kieran Wynne, Sarah Kellihier, Karl Ewins, Ellen O’Rourke, Karl Egan, Eric Moran, Georgi Petrov, Ashish Patel, and Áine Lennon collected data and performed experiments; Luisa Weiss, Paulina B. Szklanna, Fionnuala Ní Áinle, and Patricia B. Maguire analyzed data; Luisa Weiss, Shane P. Comer, Fionnuala Ní Áinle, and Patricia B. Maguire wrote the paper. All authors approved the final manuscript.

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**SUPPORTING INFORMATION**

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

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