

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2024 September ; 44(9): 2038–2052. doi:10.1161/ATVBAHA.124.320902.

Thrombin generation is associated with extracellular vesicle and leukocyte lipid membranes in atherosclerotic cardiovascular disease

Majd B Prottý^{1,*}, Victoria J Tyrrell¹, Keith Allen-Redpath², Shin Soyama², Ali A Hajeyah¹, Daniela Costa¹, Anirban Choudhury³, Rito Mitra⁴, Amal Sharman², Parveen Yaqoob², P Vince Jenkins¹, Zaheer Yousef⁴, Peter W Collins¹, Valerie B O'Donnell^{1,*}

¹Systems Immunity University Institute, Cardiff University, Cardiff, CF14 4XN, UK.

²Department of Nutritional Sciences, University of Reading, Reading, RG6 6AP, UK.

³Morrison Cardiac Centre, Swansea Bay University Health Board, Swansea, SA6 6NL, UK.

⁴Department of Cardiology, University Hospital of Wales, Cardiff, CF14 4XW, UK.

Abstract

Background—Clotting, leading to thrombosis, requires interactions of coagulation factors with the membrane aminophospholipids (aPL) phosphatidylserine (PS) and phosphatidylethanolamine (PE). Atherosclerotic cardiovascular disease (ASCVD) is associated with elevated thrombotic risk which is not fully preventable using current therapies. Currently, the contribution of aPL to thrombotic risk in ASCVD is not known. Here, the aPL composition of circulating membranes in ASCVD of varying severity will be characterized along with the contribution of external-facing aPL to plasma thrombin generation in patient samples.

Methods—Thrombin generation was measured using a purified factor assay on platelet, leukocyte and extracellular vesicles (EV) from patients with ACS (n = 24), stable coronary artery disease (CAD, n = 18), risk factor positive (RF, n = 23) and compared with healthy controls (HC, n = 24). aPL composition of resting/activated platelet and leukocytes, and EV membranes was determined using lipidomics.

Results—External facing aPL were detected on EV, platelets and leukocytes, elevating significantly following cell activation. Thrombin generation was higher on the surface of EV from ACS patients than HC, along with increased circulating EV counts. Thrombin generation correlated significantly with externalized EV PS, plasma EV counts and total EV membrane surface area. In contrast, aPL levels and thrombin generation from leukocytes and platelets were not impacted by disease, although circulating leukocyte counts were higher in patients.

Conclusions—The aPL membrane of EV supports an elevated level of thrombin generation in patient plasma in ASCVD. Leukocytes may also play a role although the platelet membrane did

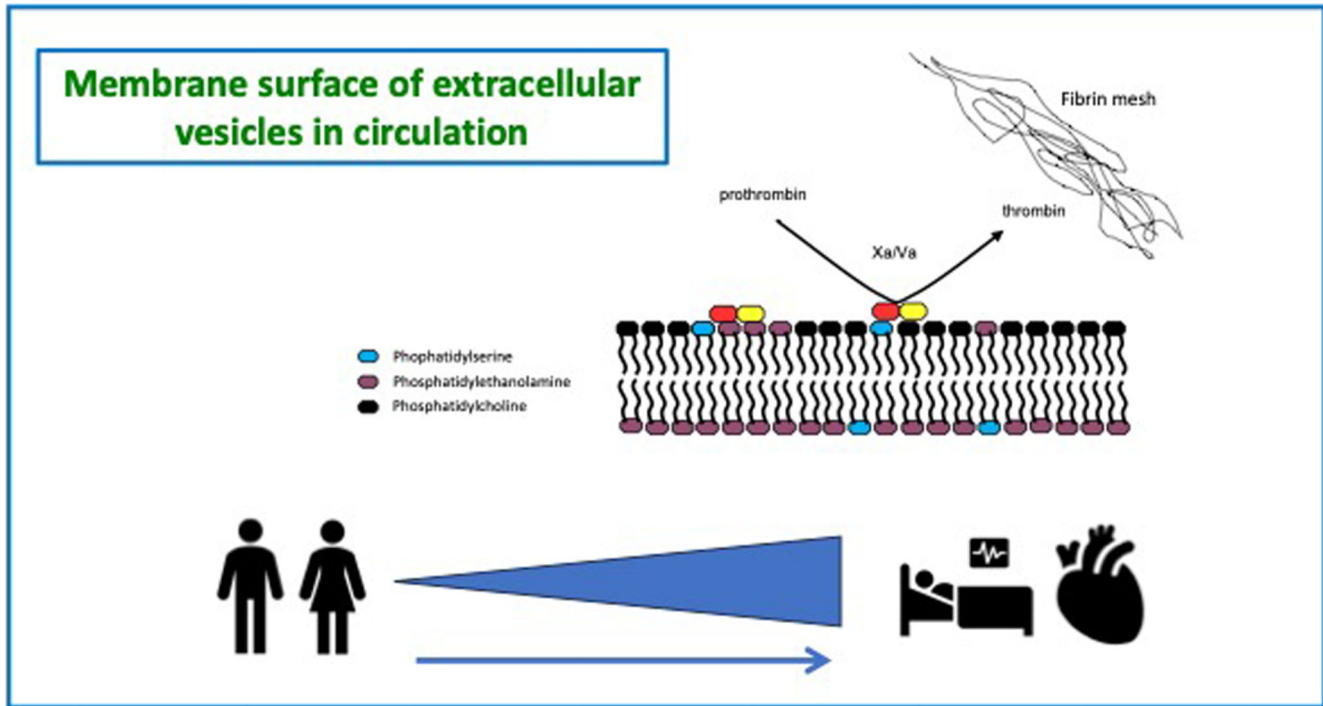
This work is licensed under a [BY 4.0 International license](https://creativecommons.org/licenses/by/4.0/).

* **Address for correspondence:** Prof Valerie O'Donnell or Dr Majd Prottý, Systems Immunity University Research Institute, Cardiff University, Heath Park, Cardiff, CF14 4XN, United Kingdom, o-donnellvb@cardiff.ac.uk or prottym3@cardiff.ac.uk .

Disclosures: None

not seem to contribute. Targeting EV formation/clearance and developing strategies to prevent the aPL surface of EV interacting with coagulation factors represents a novel anti-thrombotic target in ASCVD.

Abstract



Keywords

lipidomics; thrombosis; phospholipids; acute coronary syndrome

Non-Standard Abbreviations

ASCVD	Atherosclerotic cardiovascular disease
EV	Extracellular vesicles
aPL	Aminophospholipids
HC	Healthy controls
RF	Risk factor positive
ACS	Acute coronary syndrome
CAD	Coronary artery disease
TF	Tissue factor
PS	Phosphatidylserine

PE	Phosphatidylethanolamine
PL	Phospholipid
LC/MS/MS	Liquid chromatography tandem mass spectrometry
TAT	Thrombin-anti thrombin complex

Introduction

Atherosclerotic cardiovascular disease (ASCVD) refers to a spectrum of conditions ranging from acute coronary syndrome (ACS), stable coronary artery disease (CAD), and patients with risk factors that have not yet developed obstructive coronary artery stenosis (RF). Worldwide, ASCVD is responsible for the death of more than 9 million people annually, with an estimated prevalence of approximately 200 million^{1,2}. The most severe form of ASCVD is ACS which is triggered by atherosclerotic plaque rupture leading to activation and recruitment of platelets and leukocytes along with upregulation of tissue factor (TF) expression³⁻¹⁰. This activates coagulation, forming an occlusive arterial thrombus driving ischemia and infarction¹¹. Despite standard treatment with anti-platelet agents, the rates of subsequent strokes, myocardial infarction and cardiovascular death exceed 10% in the first-year post ACS diagnosis^{12,13}. This indicates that there are likely to be other modifiable factors involved in ASCVD beyond platelet activity¹²⁻¹⁴.

Thrombin generation requires a procoagulant phospholipid (PL) membrane to allow the assembly of the “prothrombinase” (Factor Xa/Va) complex and other Gla-domain containing coagulation factors¹⁵. This can be provided by the external surface of activated platelets, leukocytes and extracellular vesicles (EV)^{16,17}. Resting platelet and leukocyte membranes are comprised primarily of phosphatidylcholine (PC) on the external side, with the aminophospholipids (aPL) phosphatidylethanolamine (PE) and phosphatidylserine (PS) being internally facing¹⁸. The binding of coagulation factors requires a specific phospholipid (PL) composition which includes the electronegative headgroup of PS, supported by PE, and is dependent on the presence of calcium ions^{19,20}. Native PC does not facilitate the binding of coagulation factors and so resting platelets or leukocytes provide minimal support for coagulation reactions. During inflammation or acute trauma/bleeding challenge, platelets and leukocytes become activated, leading to a calcium-dependent translocation of PS/PE to the outside of the cell, mediated by scramblase²¹. Inflammation also leads to the release of aPL-rich EV. Together, this exposure of aPL to the circulation leads to interactions with coagulation factors via calcium which are essential for effective coagulation *in vivo*²²⁻²⁴.

Whether the enhanced thrombotic risk in ASCVD is related to altered levels of aPL-mediated thrombin generation on the surface of blood cells is currently unknown. Previous studies have focused on differences in coagulation factor amounts and activity, but there have been no studies focusing on the contribution of the PL membrane to thrombin generation independent of tissue factor or plasma content²⁵. To determine this, we characterized the aPL membrane composition in platelets, leukocytes and EV in patients with ACS, CAD and RF. We assessed the ability of these membranes to support coagulation *in vitro* compared with healthy controls (HC) using a thrombin generation assay that

employs purified coagulation factors independent of tissue factor^{26–29}. This approach allowed the contribution of the pro-coagulant membrane to coagulation to be determined specifically.

TOP

Methods

Study Participants for clinical cohort (blood samples)—Clinical cohort:

Participants were recruited from Cardiff University and Cardiff and Vale University Health Boards. Ethical approval was from Health and Care Research Wales (HCRW, IRAS 243701; REC reference 18/YH/0502). Age and sex-matched individuals were recruited into one of the following four groups: (i) *Acute Coronary Syndrome (ACS)*: Participants were identified on in-patient cardiology wards using diagnostic tests (ischemic ECG changes, raised troponin level above normal laboratory defined range) and clinical assessment by the cardiology team. All were recruited within 48 hours of the index event prior to any revascularization/angioplasty. (ii) *Significant coronary artery disease (CAD)*: Patients attending for an elective coronary angiogram to assess for symptoms of stable angina in the absence of a history of ACS. Coronary angiography demonstrated lesions requiring revascularization on anatomical/physiological criteria as defined by guidelines from the European society for cardiology (ESC, 2018)³⁰. (iii) *Risk-factor controls with no significant CAD (RF)*: This group includes patients attending for a diagnostic coronary angiogram with risk factors for ischemic heart disease (a clinical diagnosis of hypertension requiring therapy, diabetes types 1 or 2, hypercholesterolemia [total cholesterol > 6 mmol/L], smoking, chronic kidney disease stage 3 or more, or combination thereof) but whose coronary angiogram demonstrates no significant coronary artery disease, defined as not requiring revascularization on anatomical/physiological criteria as per the ESC 2018 guidelines³⁰. (iv) *Healthy controls (HC)*: Participants had no significant history for ischemic heart disease or its risk factors, were never-smokers, and were not on anti-platelet agents, anti-coagulants or statins. They were identified from the workplace or were volunteers from partner studies such as ‘HealthWise Wales’³¹. Clinical characteristics are in Table S1. Inclusion criteria were aged 18 years or over, acute coronary syndrome in ACS group, and no history of ACS in the others. Exclusion criteria were: diagnosis of infective endocarditis or atrial fibrillation, or inability to consent to study. Overall, 90 participants were recruited: HC, n = 24, RF, n = 23, CAD, n = 19, ACS, n = 24. Blood samples were collected by peripheral venepuncture as outlined below, by one individual and all samples were transferred to the laboratory within 10 min. The study design is summarized in Figure S1. Blood cell counts were not available for 2 CAD patients. In our human study, numbers of males and females in patient groups were not significantly different, when compared to healthy controls, using Fisher’s exact test (Table S1). Data from males and females were combined and sex differences were not determined in the study due to group sizes being too small. Primary analyses were pre-planned, not post-hoc and p values are specified on figures where < 0.05. Power calculations were not performed due to the lack of relevant primary data with which to perform them.

Platelet isolation—Whole blood was taken from using a 21G butterfly needle into a 50 ml syringe containing acidified citrate dextrose (ACD; 85 mM trisodium citrate, 65 mM citric acid, 100 mM glucose) at a ratio of 8.1 parts whole blood to 1.9 parts ACD, as described previously²⁶, and centrifuged at 250 *g* for 10 min at 20 °C. The platelet-rich plasma was collected and centrifuged at 1000 *g* for 8 min at 20 °C. Platelet poor plasma was removed and retained for extracellular vesicle isolation. The platelet pellet was resuspended in Tyrode's buffer (134 mM NaCl, 12 mM NaHCO₃, 2.9 mM KCl, 0.34 mM Na₂HPO₄, 1.0 mM MgCl₂, 10 mM Hepes, 5 mM glucose, pH 7.4) containing ACD (9:1, v/v). The platelets were washed by centrifuging at 1000 *g* for 8 min at 20 °C then resuspended in Tyrode's buffer at $2 \times 10^8 \cdot \text{ml}^{-1}$. Platelets were activated at 37 °C in the presence of 1 mM CaCl₂, 0.2 unit·ml⁻¹ thrombin (Sigma Aldrich).

Leukocyte isolation—Leukocytes were isolated from 20 ml citrate-anticoagulated whole blood as described previously²⁶. Briefly, 20 ml of blood was drawn using a 21G butterfly needle into a 50 ml syringe containing 4 ml of 2 % citrate and 4 ml of Hetasep (Stem Cell Technologies) and allowed to sediment for 45 minutes. The upper plasma layer was recovered and centrifuged at 250 *g* for 10 min at 4 °C. The pellet was resuspended in ice-cold 0.4 % trisodium citrate/PBS and centrifuged at 250 *g* for 5 min at 4 °C. Erythrocytes were removed by hypotonic lysis (0.2 % hypotonic saline before being neutralized with a PBS wash. Leukocytes were resuspended in Krebs buffer (100 mM NaCl, 48 mM HEPES, 5 mM KCl, 1 mM sodium dihydrogen orthophosphate dihydrate and 2 mM glucose) at $4 \times 10^6/\text{ml}$. For activation, 4×10^6 leukocytes were incubated at 37 °C with 10 μM A23187 and 1 mM CaCl₂, for 30 min, prior to lipid extraction.

Extracellular vesicle (EV) isolation for clinical assay—Methods were adapted from recent literature and guidelines^{32,33}. Platelet poor plasma generated as above was centrifuged at 1000 *g* for 10 min at 20 °C to generate platelet-free plasma (PFP). 1 ml PFP was snap frozen on dry ice and stored at -80 °C for quantification at a later date as below. For each donor's plasma, 6 x 1 ml PFP aliquots were centrifuged at 16,000 *g* for 30 min at 20 °C. 750 μl was removed from each aliquot, and 750 μl of modified Tyrode's buffer was added to the pellet, which was gently resuspended using a pipette. Following a second centrifugation as above, 950 μl was removed. 50 μl modified Tyrode's buffer was added to the pellet to gently resuspend and recover the EV-rich fraction. EV fractions were pooled to generate one isolate per donor. Of this, 250 μl was used for lipidomics, and 3 x 20 μl for prothrombinase assays.

Extracellular vesicle (EV) quantification for clinical assay—EV quantification was performed by thawing one aliquot of PFP per patient, of which 500 μL was passed through size-exclusion chromatography iZON qEV columns (Izon Science Ltd, UK) to recover particles and vesicles between 70 nm to 1000 nm in diameter as outlined in the manufacturers information. The eluting EV-rich fractions were collected and analyzed using nanoparticle tracking on a NanoSight 300 (Malvern, UK) equipped with a sensitive sCMOS camera and a 488 nm blue laser, to generate an EV count and size distribution for all participants. For two samples, a measure of count and size could not be obtained due to turbidity.

ELISA—Commercially available ELISA kits were purchased (Abcam, UK) for the measurement of apolipoprotein B (ab190806), D-Dimer (ab260076) and human thrombin anti-thrombin complexes (ab279724) on frozen plasma samples. These were quantified on a plate reader for absorbance. For all available information on the kits and antibodies used, please see the following links: [https://www.abcam.com/ps/products/190/ab190806/documents/Human-Apolipoprotein-B-elisa-kit-protocol-book-v3-ab190806%20\(website\).pdf](https://www.abcam.com/ps/products/190/ab190806/documents/Human-Apolipoprotein-B-elisa-kit-protocol-book-v3-ab190806%20(website).pdf) [https://www.abcam.com/ps/products/260/ab260076/documents/Human-D-Dimer-ELISA-Kit-protocol-book-v3a-ab260076%20\(website\).pdf](https://www.abcam.com/ps/products/260/ab260076/documents/Human-D-Dimer-ELISA-Kit-protocol-book-v3a-ab260076%20(website).pdf)

Testing for lipoprotein contamination EV isolated by SEC—Twelve male and female subjects (females=10, males=2) (aged 22-37y) were recruited from the University of Reading. All subjects gave informed consent. Ethical approval for the study was obtained from the School of Chemistry, Food and Pharmacy Ethics Committee at the University of Reading (Study number 17/17) and conducted according to the guidelines laid down in the Declaration of Helsinki. The inclusion criteria comprised the following: Age range 18-65 y, nonsmoker, haemoglobin 115 g/l for women and 130 g/l for men, total cholesterol < 5 mmol/l, TAG 0.4-1.5 mmol/l, no disclosed history of drug or alcohol abuse, no illness or disease requiring medication (excluding HRT, oral contraceptive, and thyroxine replacement therapy). Participants were excluded if unwell or on any prescribed medication and were asked to avoid exercise, alcohol and fatty meals the day before the experimental visit, consuming a low-fat meal the evening before the visit. Participants attended the Hugh Sinclair Unit of Human Nutrition following a 12 h overnight fast and a baseline fasting blood sample was taken before consuming a high fat test meal consisting of two all-butter croissants containing 24 g fat, 10.6 g protein and 48 g carbohydrate. A second blood sample was collected 4 h later, corresponding to the point at which postprandial lipaemia reaches a peak. Venous blood samples were drawn into citrated tubes, inverted 4 times, and processed immediately. Samples were centrifuged at 1500 x g at room temperature for 15 min to remove larger cells and cellular debris. Further centrifugation at 13000 x g for 2 min at room temperature produced PFP, which was aliquoted and stored at -80°C for further analysis.

- (i) *Isolation of EVs using SEC.* PFP (0.5 ml) was thawed at room temperature on a sample roller and loaded onto a qEV original column (Izon, Oxford, UK), which had been pre-flushed with 30 ml phosphate-buffered saline (PBS). A further 5 ml PBS was passed through the column to elute EVs based on their size and 0.5 ml fractions were collected.
- (ii) *Lipoprotein isolation.* Density gradient centrifugation was performed on baseline and postprandial PFP to isolate chylomicrons, VLDL-1 and VLDL-2, and the fractions were stored at -20°C in the presence of apo B48 preservative (5% v/v) for later analysis, as described in³⁴.
- (iii) *Assessment of contamination of EV fractions with apoB48 and apoB100.* To determine whether EV fractions prepared from PFP by SEC were contaminated with chylomicrons or VLDL, EV fractions prepared from both baseline and postprandial samples of PFP were subjected to ELISA for Apo B48 (CM) and Apo B100 (VLDL). The presence of CM was evaluated using the Human Apolipoprotein B48 ELISA kit purchased from ElabScience (Houston, USA)

and the presence of VLDL was investigated using the human apolipoprotein B100 ELISA kit. (SIGMA-Aldrich, Saint Louis, USA). The ELISA tests were performed according to the manufacturer's instructions and read on a plate reader (Spark, Tecan, UK).

- (iv) *Assessment of the potential co-isolation of lipoproteins prepared by density gradient centrifugation with EVs.* CM, VLDL-1 and VLDL-2 fractions prepared by density gradient centrifugation (as described above) were subjected to SEC to determine whether there was co-elution of lipoproteins and EVs. Fractions were analysed using nanoparticle tracking analysis.

Prothrombinase assay—Resting platelets (4×10^6), resting leukocytes (8×10^4) and plasma EV isolates (20 μ L) were added in triplicate to a 96-well half-area flat bottom clear plate (Greiner, Austria). Next, a mix of recombinant factor Xa (Fxa) (50 nM, Enzyme Research Laboratories, UK), factor Va (Fva) (15 nM, Haematologic/Cambridge Bioscience, UK), factor II (FII) (1 μ M, Enzyme Research Laboratories, UK) and CaCl_2 (5 mM) in prothrombinase buffer (20 mM Tris, 150 mM NaCl, 0.05% BSA w/v) was added to the wells and the reaction allowed to proceed for 5 min at 21 °C, before being quenched with an excess of EDTA (7 mM final concentration). Thrombin (FIIa) activity was measured on a plate reader using a chromogenic substrate S-2238 (Enzyme Research Laboratories, UK). A graphical depiction of the assay is shown in Figure S2.

Lipid biotinylation, extraction and analysis for aPL—To determine the amounts of PE and PS on the external leaflet of cell membranes, total and external aPL were measured as described previously³⁵. Briefly, 0.2 mL of platelets (4×10^7), leukocytes (8×10^5) or EV isolated as described above were incubated with 20 μ L of 20mM NHS-biotin for 10 min at 21 °C to label total aPL. In the case of externalized aPL, samples were incubated with 86 μ L of 11mM EZ-Link sulfo-NHS-biotin for 10 min at 21 °C followed by 72 μ L of 250 mM of L-lysine for 10 min at 21 °C. The final volumes were made up to 0.4 mL with phosphate-buffered saline (PBS). Lipids were extracted by adding samples to 1.5 mL chloroform:methanol (1:2) containing 10 ng internal standards (biotinylated 1,2-dimyristoyl-PE and -PS, generated as in Thomas et al³⁵) to give a solvent:sample ratio of 3.75:1, as described previously³⁵. Following vortexing and centrifugation (400 g, 5 mins), lipids were recovered in the lower chloroform layer, which was dried under vacuum. Samples were analyzed for aPL using LC/MS/MS. For this, samples were separated on an Ascentis C-18 (5 μ m 150 mm \times 2.1 mm) column (Sigma Aldrich, USA) with an isocratic gradient (methanol, 0.2 % w/v $\text{NH}_4\text{CH}_3\text{CO}_2$) at a flow rate of 400 μ L/min. Products were analyzed in MRM mode on a Q-Trap 4000 instrument (Applied Biosystems, UK) by monitoring precursor-to-product ion transitions in negative ion mode (Table S2). The peak area for the analytes was integrated and normalized to the internal standards. Limit of quantitation (LOQ) is defined as signal:noise of 5:1 with at least 6 data points across a peak. For quantification, standard curves were generated using biotinylated PS and PE³⁵. Chromatograms of biotinylated PS and PE species as detected in representative participant samples can be seen in Figures S3,4, respectively. For 5 patient extracts processed on the same day, internal standard could not be detected during subsequent LC/MS/MS and they were excluded. In a further 8 samples, while conducting LC/MSMS analysis of the full cohort samples, we identified that a batch

of NB used for this subset of samples had been inactive. SNB used for those samples was active so externalized but not total PE/PS levels were determined for these samples. Full data on all sample numbers are included in legends for clarity.

Statistical analysis—Statistical significance was determined using one way ANOVA with Tukey Post Hoc test (astatsa.com). For platelets and leukocytes, resting or activated samples were compared separately. Correlation analysis utilized Pearson's correlation for linear dependence between variables. The cut-off value chosen for test significance was a p-value <0.05. Box plots were drawn in Excel (Microsoft, USA) with edges indicating the interquartile range (IQR), the line inside the box indicating the median, and whiskers indicating 1.5 times the IQR. For generation of heatmaps, samples were averaged within their groups, and a log10 was applied to lipid amounts (ng) normalized to cell count or tissue weight (mg) for each lipid to allow row-wise and column-wise comparison. Next, lipid measurements were plotted as intensity values using the 'pheatmap' package in the R coding environment (v3.6.2, open source) with lipid hierarchical clustering. Intensity levels were represented by a colour gradient ranging from blue (very low levels or absent) to red (high levels) with variations in between. Graphical illustrations of assays, designs and pathways were carried out using the online platforms draw.io and biorender.com (premium subscription). For lipidomics data statistical analysis, missing values where lipids were below LOQ were replaced with 50% of the assay LOQ value. If any lipid was missing more than 50 % in a sample set, it was not analysed. Where values were imputed, they are shown in red in the Supplementary Dataset.

Results

EV-containing plasma from ACS patients supports elevated thrombin generation, driven by higher EV counts in disease

EV fractions isolated using either centrifugation or SEC were found to be free of lipoproteins as measured using the ab190806 human apolipoprotein B ELISA kit (Abcam, UK) (Figure S5 A), or by monitoring lipoprotein elution from SEC (Figure S5 B-F). Here, When PFP was subjected to SEC, EVs eluted in Fractions 7-9. However CM (apo B48) and VLDLs (apo B100) in PFP only began to elute from Fraction 11 onwards (Figure S5 B,C), demonstrating that lipoproteins elute later than EVs, with little cross-contamination. This is consistent with lipoproteins having a smaller diameter than EVs. Lipoproteins isolated first by density gradient centrifugation and then subjected to SEC did not elute in EV enriched fractions (Fractions 7-9) and began to elute in the later fractions (Figure S5 D-F). Specifically, CM isolated from fasted plasma eluted after fraction 12 and those isolated from postprandial plasma eluted after fraction 10, indicating that the postprandial CM population was larger in diameter, but still distinct from the EV population. The VLDL-1 population eluted after Fraction 14 and the VLDL-2 population eluted after fraction 16, indicating the progressively smaller diameters of these lipoproteins.

In an in vitro system using purified Fxa, Fva and FII, EV-containing plasma from patients with ACS stimulated significantly higher thrombin generation than those from HC, while there was a higher trend for RF and CAD (Figure 1 A). EV were isolated from a fixed

volume (6 mL) of plasma, thus, particles were quantified using nanoparticle tracking analysis. There was a trend for higher EV counts in plasma from all patient groups compared with HC, which was significantly higher for CAD (Figure 1 B). Furthermore, there was a significant weak positive correlation between thrombin generation and EV counts (Figure 1 C). The mean EV vesicle diameter was significantly smaller in RF and CAD, and slightly smaller in ACS, compared with HC (Figure S6). As coagulation takes place on the surface of vesicles, we next calculated the EV total surface area (vesicle area as a sphere calculated as $4\pi r^2 \times \text{EV counts}$) in plasma. A trend was seen for higher EV total surface areas in patient groups although they showed greater overall variability (Figure 1 D). Thrombin generation correlated significantly with EV total surface area indicating a direct relationship between EV membrane and coagulation (Figure 1 E). Overall, this indicates that EV from ASCVD patient groups (RF, CAD and ACS) tend to be smaller than HC, but due to higher counts, the overall EV surface area is elevated in disease groups and will directly contribute to elevated thrombin generation observed. Confirming this idea, we normalized thrombin generation to either EV counts or surface area and found that this abolished the differences between groups (Figure 1 E-F).

Characterisation of aPL molecular species in EV shows levels tend to be higher in disease groups

The aPL composition of EV has not been described before, hence total and external PS and PE species were next characterized using LC/MS/MS. In this assay, external facing aPL are derivatized using the cell impermeable biotinylation reagent, sulfo-NHS-biotin. Total aPL is instead derivatized using the cell-permeable form, NHS-biotin³⁵. Derivatized aPL are then detected using LC/MS/MS, based on a mass shift of +226 a.m.u. from the native lipid. This method was previously used to determine the molecular species and amounts of PS and PE on the surface of platelets from healthy donors²⁷. The most abundant species detected were: PE P-16:0_20:4, PE 18:0_20:4, PE P-18:0_20:4, PS 18:0_18:1 and PS 18:0_20:4 (Figure 1 G, Figure S7 A), as previously shown in healthy platelets²⁷. Externalized PS and PE comprised the same molecular species, with the most abundant isomers being detected in higher amounts on the outside (Figure 1 H, Figure S7 B). This indicates an absence of selectivity for any particular aPL isomers to be present on the outer side of EV.

EV from plasma of CAD patients contained higher levels of total PS and total PE, with a trend for higher levels in all patient groups, compared to HC (Figure 2 A,B), however, once data were normalized to EV counts, this disappeared indicating that it was due to higher EV being present in plasma from ACS and CAD patients (Figure 2 C,D). External PS amounts significantly correlated with thrombin generation on EVs in line with a functional involvement in driving coagulation (Figure S8 A). Externalized PS showed a non-significant trend to be higher in all patient groups versus HC, although externalized PE was similar (Figure 2 E,F). Once adjusted by EV count, externalized aPL trends disappeared (Figure 2 G,H). These data suggest that EV drive coagulation via their external PS which may be somewhat elevated in ASCVD groups compared with HC.

Thrombin generation in leukocytes from ACS patients is slightly higher than HC

Next, we characterized the pro-coagulant membrane on circulating white cells from patient groups, also comparing their ability to support thrombin generation with PE/PS content. Resting leukocytes (8×10^4) from patients showed a trend to support higher thrombin generation than HC, in particular for ACS patients (Figure 3 A). In ASCVD groups, there were significantly higher circulating leukocyte counts as measured by the hospital clinical laboratory, with worsening disease, although all cell counts were within normal range (Table S1). To test the potential impact of cell count *in vivo*, thrombin generation was normalized by circulating leukocyte count, since a higher count in disease could further impact thrombin generation. This further demonstrated an upward trend with higher amounts of thrombin predicted to be generated in ACS compared with RF (Figure 3 B). Leukocyte counts had not been obtained for HC, as volunteers were outside the hospital system, so direct comparison with this group was not possible.

Leukocytes externalize aPL on activation, although molecular composition is unchanged in coronary artery disease

The aPL composition of leukocyte membranes is currently unknown. To characterize this, and determine the impact of ASCVD, total and external molecular species of aPL were quantified in leukocytes, both before and after calcium ionophore activation. Activation reveals the potential of the cells, when fully activated, to externalize aPL, while resting cells are more comparable with circulating cells. Three PS and five PE species were detected, with all PE containing 20:4, and of a similar composition to EV (Figure 3 C,D, Figure S9 A). Total PE and PS was similar for all groups, and this was not impacted by activation (Figure 4 A,B Figure S9 B). In contrast, following ionophore activation, leukocytes from all groups externalized aPL (Figure 4 C,D). The most abundant aPL isomers were detected in higher amounts on the outside indicating a lack of selectivity for any specific aPL isomer to be externalized in leukocytes (Figure 4 C,D, Figure S9 A). Externalized aPL species or amounts showed only small differences between patient groups, and there was no significant correlation between the amount of thrombin generated on the surface of resting leukocytes and externalized PS and PE amounts (Figure 4 C,D, Figure S8 B). These data indicate that the elevated thrombin generation on the surface of leukocytes in ACS compared with HC is unlikely to be related to levels of externalized aPL.

Thrombin generation on the surface of resting platelets was similar for all groups

Next, the ability of washed platelets from patient groups to support prothrombinase activity was tested. Thrombin generation was stimulated by platelets from all groups tested, but there were no significant differences seen (Figure 5 A). Furthermore, platelet counts were similar between disease groups with all being within the normal range (Table S1). Since aspirin was highly prescribed among patients (Table S1), we next tested whether this drug had an impact on platelet driven thrombin generation in our assay, using platelets from healthy control volunteers. No effect of *in vitro* aspirin was seen, indicating that supplementation was not reducing the ability of platelet membranes to stimulate thrombin generation (Figure S8 D). Overall, this data suggests that platelet membrane-driven thrombin generation is not responsible for increased thrombotic risk in ACS.

Platelets externalize aPL on activation, with an unchanged molecular composition between disease groups and healthy controls

Next, the pro-coagulant PL composition of platelets was determined using LC/MS/MS. The aPL species detected were as previously found in healthy control platelets, namely: PE P-16:0_20:4, PE P-18:1_20:4, PE 18:0_20:4, PE P-18:0_20:4, PE 18:0_18:1, PS 18:1_18:1, PS 18:0_18:1 and PS 18:0_20:4, as previously shown²⁷ (Figure 5 B, Figure S10 B). Following thrombin activation, platelets from all groups externalized aPL with the most abundant isomers detected in higher amounts on the outside (Figure 5 C, Figure S10 A).

Total PS and PE levels were somewhat higher in platelets from the CAD group compared to HC in resting and activated conditions (Figure 6 A,B). Following thrombin activation, externalized PS and PE rose compared to resting platelets but there were no significant differences between clinical groups and HC (Figure 6 C,D). Thus, disease did not result in major changes in either the amounts or molecular species of aPL externalized by platelets. Furthermore, there was no correlation between the amount of thrombin generated and externalized PS and PE amounts on the platelet membrane surface (Figure S8 C). In summary, platelets from patients with disease did not have higher PE/PS externalization or elevated thrombin generation capacity suggesting that changes in platelet aPL do not significantly contribute to the higher thrombotic risk seen in cardiovascular disease.

Levels of TAT and D-Dimer in plasma are not altered in patient groups

Plasma D-Dimer and thrombin anti-thrombin (TAT) complexes were measured for all participants. There were no differences between groups in D-Dimer levels and for most participants, TATs were undetectable (Figure S11 A,B). Additionally, there was no significant correlations between D-Dimers and TATs (Figure S11 C) nor with the amount of thrombin generated on the surface of EV, platelets or leukocytes (Figure S11 D). Finally, the levels of plasma D-Dimer and TAT showed no significant correlations with total or externalized aPL in EV, platelets or leukocytes (Figures S12,13).

Discussion

Thrombin generation on the plasma membrane surface relies on the presence of procoagulant lipids, primarily native PS and PE, but up to now it was not known whether thrombin generation or native aPL are altered in arterial thrombosis. This is important as it may influence the procoagulant status of patients with this condition and lead to further thrombotic complications^{12,13}. Here, thrombin generation and the aPL composition of circulating blood cells and EV from patients with ASCVD was characterized. Our data suggest that EV and leukocyte membranes may contribute to increased thrombotic tendency of patients with cardiovascular disease. In contrast, a role for platelets in driving membrane-dependent coagulation was not revealed. This has implications for the clinical management of ACS which currently does not target leukocyte or EV membrane compartments therapeutically for reducing thrombotic risk.

The assay employed in this study to assess thrombin generation is TF independent and no external PL was added. The means that the assay evaluates the role that the procoagulant

membrane PL surface has on prothrombinase activity and is independent of changes in the levels of the patient's coagulation factors. Whilst there were minimal differences in aPL amounts on the surface of EV and leukocytes between disease groups and HC as characterized by LC/MS/MS, the higher count of EV in plasma provided more surface area for the prothrombinase reaction to take place which in turn led to more thrombin generation. The same is likely for leukocytes which were found to be higher in patients with ACS compared with RF. Together, these results imply that a simple abundance of circulating procoagulant surfaces is sufficient to alter the amount of thrombin generation in resting states. Targeting EV clearance or shielding the circulation from the aPL membrane may therefore present a novel angle to reduce coagulation reactions in this thrombotic condition.

The use of LC/MS/MS to characterize aPL in platelets, leukocytes and EV has not been employed in arterial thrombosis patients prior to this study. Additionally, the external aPL lipidome in leukocytes and EV has not been previously characterized with LC/MS/MS. The majority of the literature on aPL trafficking and detection utilizes a flow cytometry-based method which relies on aPL-binding fluorescent probes³⁶⁻³⁸. The commonest of these is annexin V-FITC, which can bind to either PS or PE in the presence of calcium^{37,38}. There are a number of limitations to this method, the main one being its non-quantitative nature. The binding of annexin V probes to cells and EV during flow cytometry exhibits rapidly saturated kinetics, likely as a result of steric hindrance preventing additional aPL from binding to this large protein²⁷. Consequently, whilst it is feasible to count annexin V⁺ cells and EV using this method, it is not possible to quantify how much aPL is on the surface, distinguish between PS and PE or know what molecular species are exposed. This is relevant since the procoagulant activity of aPL can be influenced by fatty acyl composition, with an impaired ability of PE comprising shorter FA chains to support coagulation²⁷. Mass spectrometry with biotin derivatization allows the quantitative analysis of aPL molecular species, distinguishing between external and total aPL amounts³⁵. Our study provides a first characterization of PS and PE species present on circulating membrane surfaces using an assay that distinguishes between total and externalized aPL³⁵. The findings build on the current understanding of aPL externalization which had previously been reported in ACS patients using non-quantitative techniques with annexin V/lactadherin binding³⁹.

Several studies have described higher numbers of EV in patients suffering from arterial thrombosis and its risk factors⁴⁰⁻⁴⁶. Hypertensive patients have more circulating EV which correlate with their blood pressure readings and are thought to be generated as a consequence of higher shear⁴⁰. Similarly, diabetes mellitus is associated with higher levels of EV released as a consequence of stimuli such as advanced glycation products and oxidative stress, with a higher procoagulant phenotype in patients with poor glycemic control^{41,42}. Patients with dyslipidemias have upregulated EV levels due to LDL-induced membrane blebbing⁴³. Indeed, the development of atherosclerosis may be influenced by EV, generated by mechanisms described above, which alter the profile of adhesion molecules on endothelial cells and promote monocyte transmigration and vascular inflammation^{47,48}. In addition, the presence of high amounts of EV within the atherosclerotic plaque may contribute to the thrombotic process that follows plaque rupture⁴⁹. This may implicate EV in ACS where elevated numbers were demonstrated in comparison to CAD patients⁴⁴⁻⁴⁶, and shown to positively correlate with high-risk coronary angiographic features⁵⁰. Previous

studies characterizing plasma from ASCVD patients showed that the highest proportion of EVs in these groups is platelet-derived (40-50%), followed by endothelial-derived (20-25%) then leukocyte-derived (5-10%)⁵¹. Neutrophil derived EV comprise a relatively low %, being around 5% in ACS and CAD, versus less than 2% in HC^{51,52}. Nevertheless, beyond their proposed use as biomarkers and their elevated amounts, the exact role and regulation of EV in human arterial thrombosis remains largely unstudied.

Our data shows no significant changes in platelet-supported thrombin generation or platelet PE/PS externalization. These findings suggest a lack of role for the platelet membrane in raising thrombotic risk in these patients. Notably, the purified factor-based experimental system we used to study membrane involvement does not take into account the many other potential platelet-dependent mechanisms including platelet aggregation, release of platelet-derived EVs, or secondary processes such as release of bioactive mediators that activate leukocytes (thromboxanes) or stimulate vascular and circulating blood cells to generate EVs. Indeed anti-platelet agents such as GP2b3a inhibitors work well in patients with a heavy thrombus burden supporting their central role⁵³.

Our study adds significantly to knowledge of EV biology in vascular disease, providing the first characterization of their aPL composition and how this contributes directly to thrombotic tendency. It is generally accepted that annexin V^{+ve} EV (containing externalized aPL) are procoagulant. Previous studies have concluded that a higher number of EV implies more PS exposure in the circulation which in turn could lead to a more procoagulant phenotype and our study adds significant new information to support this idea by characterizing the specific molecular species of PS and PE involved^{45,54,55}. Up to now, methods used to study PS^{+ve} EV have been limited to flow cytometry-based assays with annexin V. This does not allow accurate quantification of external aPL, individual molecular species, nor does it determine the total aPL content within membranes. LC/MS/MS and biotin derivatization allowed the mapping specific molecular species and their amounts, whilst also distinguishing between external and total membrane aPL³⁵.

In our study, TATs and D-Dimers were similar across all groups. However published literature is conflicting, with some studies showing elevations and several others not showing changes in coagulation markers in ASCVD patients⁵⁶⁻⁶². A possible explanation for no increase being seen in this cohort is that many patients were actively taking medications designed to dampen thrombosis via inhibiting platelet activation. However, considering these patients are still at elevated thrombotic risk, the characterisation of the role of additional factors such as circulating PL membranes becomes important.

The timing of any intervention targeting leukocytes or EV in ACS would require careful consideration. In this study, all ACS samples were taken within 48 h of the onset of the event, and therefore the findings reflect the acute phase. This immediate stage is associated with higher thrombotic risk⁶³. As such, it is managed with more intensive anti-thrombotic treatment in the first few days after ACS such as the addition of a low molecular-weight heparin to dual anti-platelet therapy⁶³. It is unclear however whether the higher procoagulant potential of leukocyte/EV membranes continues in the months after the acute event where persistent activation of the coagulation system have been described in ACS⁶⁴. To test this,

longitudinal studies of patients with ACS in the acute (48 hours), intermediate (6 weeks) and distant (6 months) phase would provide more information on the variation of procoagulant membrane properties over time.

Study Limitations

Whilst cells from patients with ACS may demonstrate lipidomic and biological differences, it is not possible to determine causality since it is not possible to predict when an ACS event will occur⁶⁵. Furthermore, the EV cell of origin and TF surface content is unknown. Two separate preps for EV were utilized, for the coagulation assays and EV counting. This was necessary since EV for the coagulation assay needed to be generated and used on the same day, while EV for counting could be generated later using frozen plasma. Finally, the cross-sectional design of the study on the clinical cohort and the absence of longitudinal sampling timepoints (particularly for the acute phase of ACS) may be confounded by inter-individual variations which may reduce statistical power. This, alongside the relatively small sample size, makes further validation and replication in a larger cohort advantageous.

Conclusion

In this study, the procoagulant membrane in circulating blood cells and EV was determined in patients with ASCVD. Our findings provide the first characterization of aPL in ASCVD circulating membranes and propose that higher membrane procoagulant activity in arterial thrombosis will be driven primarily by EV and leukocytes. Expanding on these findings, and testing whether interference with the procoagulant lipidome alters the observed incidence of arterial thrombosis would move the field closer towards targeting PL as therapeutic targets for the prevention of thrombosis in high-risk groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

None

Sources of funding

This work was supported by the Wellcome Trust (GW4-CAT fellowship to M.P - 216278/Z/19/Z) and the British Heart Foundation (Programme grant to P.C and V.B.O – RG/F/20/110020). VJT was supported in part by the Welsh Government/EU Ser Cymru Programme. AAH is supported by a grant from Kuwait University. PV and AS were funded by Govt of Kingdom of Saudi Arabia.

Data availability

The authors declare that all supporting data are available within the article and its online supplementary files. Raw data for individual experiments are available from the corresponding authors on reasonable request.

References

1. Vaduganathan M, Mensah GA, Turco JV, Fuster V, Roth GA. The Global Burden of Cardiovascular Diseases and Risk: A Compass for Future Health. *J Am Coll Cardiol.* 2022; 80: 2361–2371. DOI: 10.1016/j.jacc.2022.11.005 [PubMed: 36368511]
2. Roth GA, Mensah GA, Johnson CO, et al. Global Burden of Cardiovascular Diseases and Risk Factors, 1990–2019: Update From the GBD 2019 Study. *J Am Coll Cardiol.* 2020; 76: 2982–3021. DOI: 10.1016/j.jacc.2020.11.010 [PubMed: 33309175]
3. Wilson SJ, Newby DE, Dawson D, Irving J, Berry C. Duration of dual antiplatelet therapy in acute coronary syndrome. *Heart.* 2017; 103: 573–580. DOI: 10.1136/heartjnl-2016-309871 [PubMed: 28249994]
4. Mulvihill NT, Foley JB. Inflammation in acute coronary syndromes. *Heart.* 2002; 87: 201–204. [PubMed: 11847151]
5. Sager HB, Nahrendorf M. Inflammation: a trigger for acute coronary syndrome. *Q J Nucl Med Mol Imaging.* 2016; 60: 185–193. [PubMed: 27273431]
6. Rickles FR, Levin J, Hardin JA, Barr CF, Conrad ME Jr. Tissue factor generation by human mononuclear cells: effects of endotoxin and dissociation of tissue factor generation from mitogenic response. *J Lab Clin Med.* 1977; 89: 792–803. [PubMed: 321714]
7. Sernerri GG, Abbate R, Gori AM, Attanasio M, Martini F, Giusti B, Dabizzi P, Poggesi L, Modesti PA, Trotta F, et al. Transient intermittent lymphocyte activation is responsible for the instability of angina. *Circulation.* 1992; 86: 790–797. [PubMed: 1516190]
8. Mazzone A, De Servi S, Ricevuti G, Mazzucchelli I, Fossati G, Pasotti D, Bramucci E, Angoli L, Marsico F, Specchia G, et al. Increased expression of neutrophil and monocyte adhesion molecules in unstable coronary artery disease. *Circulation.* 1993; 88: 358–363. DOI: 10.1161/01.cir.88.2.358 [PubMed: 8101771]
9. Biasucci LM, D’Onofrio G, Liuzzo G, Zini G, Monaco C, Caligiuri G, Tommasi M, Rebuffi AG, Maseri A. Intracellular neutrophil myeloperoxidase is reduced in unstable angina and acute myocardial infarction, but its reduction is not related to ischemia. *J Am Coll Cardiol.* 1996; 27: 611–616. DOI: 10.1016/0735-1097(95)00524-2 [PubMed: 8606272]
10. Hansen PR. Myocardial reperfusion injury: experimental evidence and clinical relevance. *Eur Heart J.* 1995; 16: 734–740. [PubMed: 7588916]
11. Hedayati T, Yadav N, Khanagavi J. Non-ST-Segment Acute Coronary Syndromes. *Cardiol Clin.* 2018; 36: 37–52. DOI: 10.1016/j.ccl.2017.08.003 [PubMed: 29173680]
12. Komocsi A, Vorobcsuk A, Kehl D, Aradi D. Use of new-generation oral anticoagulant agents in patients receiving antiplatelet therapy after an acute coronary syndrome: systematic review and meta-analysis of randomized controlled trials. *Arch Intern Med.* 2012; 172: 1537–1545. DOI: 10.1001/archinternmed.2012.4026 [PubMed: 23007264]
13. Giri S, Jennings LK. The spectrum of thrombin in acute coronary syndromes. *Thromb Res.* 2015; 135: 782–787. DOI: 10.1016/j.thromres.2015.02.013 [PubMed: 25764910]
14. Jennings LK. Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherothrombosis. *Thromb Haemost.* 2009; 102: 248–257. DOI: 10.1160/TH09-03-0192 [PubMed: 19652875]
15. Mann KG. Biochemistry and physiology of blood coagulation. *Thromb Haemost.* 1999; 82: 165–174.
16. Furie B, Furie BC. In vivo thrombus formation. *Journal of thrombosis and haemostasis : JTH.* 2007; 5 (Suppl 1) 12–17. DOI: 10.1111/j.1538-7836.2007.02482.x
17. Falati S, Liu Q, Gross P, Merrill-Skoloff G, Chou J, Vandendries E, Celi A, Croce K, Furie BC, Furie B. Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. *The Journal of experimental medicine.* 2003; 197: 1585–1598. DOI: 10.1084/jem.20021868 [PubMed: 12782720]
18. Zwaal RF, Comfurius P, Bevers EM. Lipid-protein interactions in blood coagulation. *Biochim Biophys Acta.* 1998; 1376: 433–453. [PubMed: 9805008]

19. Krishnaswamy S, Field KA, Edgington TS, Morrissey JH, Mann KG. Role of the membrane surface in the activation of human coagulation factor X. *The Journal of biological chemistry*. 1992; 267: 26110–26120. [PubMed: 1464622]
20. Falls LA, Furie B, Furie BC. Role of phosphatidylethanolamine in assembly and function of the factor IXa-factor VIIIa complex on membrane surfaces. *Biochemistry*. 2000; 39: 13216–13222. DOI: 10.1021/bi0009789 [PubMed: 11052674]
21. Kodigepalli KM, Bowers K, Sharp A, Nanjundan M. Roles and regulation of phospholipid scramblases. *FEBS Lett*. 2015; 589: 3–14. DOI: 10.1016/j.febslet.2014.11.036 [PubMed: 25479087]
22. Tavoosi N, Davis-Harrison RL, Pogorelov TV, Ohkubo YZ, Arcario MJ, Clay MC, Rienstra CM, Tajkhorshid E, Morrissey JH. Molecular determinants of phospholipid synergy in blood clotting. *The Journal of biological chemistry*. 2011; 286: 23247–23253. DOI: 10.1074/jbc.M111.251769 [PubMed: 21561861]
23. Falls LA, Furie BC, Jacobs M, Furie B, Rigby AC. The omega-loop region of the human prothrombin gamma-carboxyglutamic acid domain penetrates anionic phospholipid membranes. *J Biol Chem*. 2001; 276: 23895–23902. DOI: 10.1074/jbc.M008332200 [PubMed: 11312259]
24. Lu J, Pipe SW, Miao H, Jacquemin M, Gilbert GE. A membrane-interactive surface on the factor VIII C1 domain cooperates with the C2 domain for cofactor function. *Blood*. 2011; 117: 3181–3189. DOI: 10.1182/blood-2010-08-301663 [PubMed: 21156843]
25. Undas A, Szuldrzynski K, Brummel-Ziedins KE, Tracz W, Zmudka K, Mann KG. Systemic blood coagulation activation in acute coronary syndromes. *Blood*. 2009; 113: 2070–2078. DOI: 10.1182/blood-2008-07-167411 [PubMed: 18931343]
26. Lauder SN, Allen-Redpath K, Slatter DA, et al. Networks of enzymatically oxidized membrane lipids support calcium-dependent coagulation factor binding to maintain hemostasis. *Sci Signal*. 2017; 10 doi: 10.1126/scisignal.aan2787
27. Clark SR, Thomas CP, Hammond VJ, Aldrovandi M, Wilkinson GW, Hart KW, Murphy RC, Collins PW, O'Donnell VB. Characterization of platelet aminophospholipid externalization reveals fatty acids as molecular determinants that regulate coagulation. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110: 5875–5880. DOI: 10.1073/pnas.1222419110 [PubMed: 23530199]
28. van Kruchten R, Mattheij NJ, Saunders C, Feijge MA, Swieringa F, Wolfs JL, Collins PW, Heemskerk JW, Bevers EM. Both TMEM16F-dependent and TMEM16F-independent pathways contribute to phosphatidylserine exposure in platelet apoptosis and platelet activation. *Blood*. 2013; 121: 1850–1857. DOI: 10.1182/blood-2012-09-454314 [PubMed: 23303820]
29. Owens AP 3rd, Mackman N. Microparticles in hemostasis and thrombosis. *Circ Res*. 2011; 108: 1284–1297. DOI: 10.1161/CIRCRESAHA.110.233056 [PubMed: 21566224]
30. Neumann FJ, Sousa-Uva M, Ahlsson A, et al. 2018 ESC/EACTS Guidelines on myocardial revascularization. *Eur Heart J*. 2019; 40: 87–165. DOI: 10.1093/eurheartj/ehy394 [PubMed: 30165437]
31. Hurt L, Ashfield-Watt P, Townson J, Heslop L, Copeland L, Atkinson MD, Horton J, Paranjothy S. Cohort profile: HealthWise Wales. A research register and population health data platform with linkage to National Health Service data sets in Wales. *BMJ Open*. 2019; 9 e031705 doi: 10.1136/bmjopen-2019-031705
32. Berckmans RJ, Lacroix R, Hau CM, Sturk A, Nieuwland R. Extracellular vesicles and coagulation in blood from healthy humans revisited. *J Extracell Vesicles*. 2019; 8 1688936 doi: 10.1080/20013078.2019.1688936 [PubMed: 31762964]
33. Coumans FAW, Brisson AR, Buzas EI, et al. Methodological Guidelines to Study Extracellular Vesicles. *Circ Res*. 2017; 120: 1632–1648. DOI: 10.1161/CIRCRESAHA.117.309417 [PubMed: 28495994]
34. Palmer AM, Nova E, Anil E, Jackson K, Bateman P, Wolstencroft E, Williams CM, Yaqoob P. Differential uptake of subfractions of triglyceride-rich lipoproteins by THP-1 macrophages. *Atherosclerosis*. 2005; 180: 233–244. DOI: 10.1016/j.atherosclerosis.2004.12.038 [PubMed: 15910848]

35. Thomas CP, Clark SR, Hammond VJ, Aldrovandi M, Collins PW, O'Donnell VB. Identification and quantification of aminophospholipid molecular species on the surface of apoptotic and activated cells. *Nat Protoc.* 2014; 9: 51–63. DOI: 10.1038/nprot.2013.163 [PubMed: 24336470]
36. Andree HA, Reutelingsperger CP, Hauptmann R, Hemker HC, Hermens WT, Willems GM. Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. *J Biol Chem.* 1990; 265: 4923–4928. [PubMed: 2138622]
37. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood.* 1994; 84: 1415–1420. [PubMed: 8068938]
38. Meers P, Mealy T. Phospholipid determinants for annexin V binding sites and the role of tryptophan 187. *Biochemistry.* 1994; 33: 5829–5837. DOI: 10.1021/bi00185a022 [PubMed: 8180211]
39. Wang L, Bi Y, Cao M, et al. Microparticles and blood cells induce procoagulant activity via phosphatidylserine exposure in NSTEMI patients following stent implantation. *Int J Cardiol.* 2016; 223: 121–128. DOI: 10.1016/j.ijcard.2016.07.260 [PubMed: 27537737]
40. Preston RA, Jy W, Jimenez JJ, Mauro LM, Horstman LL, Valle M, Aime G, Ahn YS. Effects of severe hypertension on endothelial and platelet microparticles. *Hypertension.* 2003; 41: 211–217. DOI: 10.1161/01.hyp.0000049760.15764.2d [PubMed: 12574084]
41. Min C, Kang E, Yu SH, Shinn SH, Kim YS. Advanced glycation end products induce apoptosis and procoagulant activity in cultured human umbilical vein endothelial cells. *Diabetes Res Clin Pract.* 1999; 46: 197–202. DOI: 10.1016/s0168-8227(99)00094-7 [PubMed: 10624785]
42. Sabatier F, Darmon P, Hugel B, Combes V, Sanmarco M, Velut JG, Arnoux D, Charpiot P, Freyssinet JM, Oliver C, Sampol J, et al. Type 1 and type 2 diabetic patients display different patterns of cellular microparticles. *Diabetes.* 2002; 51: 2840–2845. DOI: 10.2337/diabetes.51.9.2840 [PubMed: 12196479]
43. Llorente-Cortes V, Otero-Vinas M, Camino-Lopez S, Llampayas O, Badimon L. Aggregated low-density lipoprotein uptake induces membrane tissue factor procoagulant activity and microparticle release in human vascular smooth muscle cells. *Circulation.* 2004; 110: 452–459. DOI: 10.1161/01.CIR.0000136032.40666.3D [PubMed: 15238452]
44. Boulanger CM, Amabile N, Tedgui A. Circulating microparticles: a potential prognostic marker for atherosclerotic vascular disease. *Hypertension.* 2006; 48: 180–186. DOI: 10.1161/01.HYP.0000231507.00962.b5 [PubMed: 16801490]
45. Mallat Z, Benamer H, Hugel B, Benessiano J, Steg PG, Freyssinet JM, Tedgui A. Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. *Circulation.* 2000; 101: 841–843. DOI: 10.1161/01.cir.101.8.841 [PubMed: 10694520]
46. Werner N, Wassmann S, Ahlers P, Kosiol S, Nickenig G. Circulating CD31+/annexin V+ apoptotic microparticles correlate with coronary endothelial function in patients with coronary artery disease. *Arterioscler Thromb Vasc Biol.* 2006; 26: 112–116. DOI: 10.1161/01.ATV.0000191634.13057.15 [PubMed: 16239600]
47. Barry OP, Pratico D, Savani RC, FitzGerald GA. Modulation of monocyte-endothelial cell interactions by platelet microparticles. *J Clin Invest.* 1998; 102: 136–144. DOI: 10.1172/JCI2592 [PubMed: 9649567]
48. Burnier L, Fontana P, Kwak BR, Angelillo-Scherrer A. Cell-derived microparticles in haemostasis and vascular medicine. *Thromb Haemost.* 2009; 101: 439–451. DOI: 10.1160/TH08-08-0521 [PubMed: 19277403]
49. Mallat Z, Hugel B, Ohan J, Leseche G, Freyssinet JM, Tedgui A. Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: a role for apoptosis in plaque thrombogenicity. *Circulation.* 1999; 99: 348–353. DOI: 10.1161/01.cir.99.3.348 [PubMed: 9918520]
50. Bernal-Mizrachi L, Jy W, Fierro C, Macdonough R, Velazques HA, Purow J, Jimenez JJ, Horstman LL, Ferreira A, de Marchena E, Ahn YS. Endothelial microparticles correlate with high-risk angiographic lesions in acute coronary syndromes. *Int J Cardiol.* 2004; 97: 439–446. DOI: 10.1016/j.ijcard.2003.10.029 [PubMed: 15561331]

51. Mavroudis CA, Eleftheriou D, Hong Y, Majumder B, Koganti S, Sapsford R, North J, Lowdell M, Klein N, Brogan P, Rakhit RD. Microparticles in acute coronary syndrome. *Thrombosis Research*. 2017; 156: 109–116. DOI: 10.1016/j.thromres.2017.06.003 [PubMed: 28624718]
52. Zhang X, Hubal MJ, Kraus VB. Immune cell extracellular vesicles and their mitochondrial content decline with ageing. *Immunity & Ageing*. 2020; 17: 1. doi: 10.1186/s12979-019-0172-9 [PubMed: 31911808]
53. Tummala, R, Rai, MP. *Glycoprotein IIb/IIIa Inhibitors*. Treasure Island (FL): StatPearls Publishing; 2023.
54. Liu Y, He Z, Zhang Y, Dong Z, Bi Y, Kou J, Zhou J, Shi J. Dissimilarity of increased phosphatidylserine-positive microparticles and associated coagulation activation in acute coronary syndromes. *Coron Artery Dis*. 2016; 27: 365–375. DOI: 10.1097/MCA.0000000000000368 [PubMed: 27058313]
55. Ridger VC, Boulanger CM, Angelillo-Scherrer A, et al. Microvesicles in vascular homeostasis and diseases. Position Paper of the European Society of Cardiology (ESC) Working Group on Atherosclerosis and Vascular Biology. *Thromb Haemost*. 2017; 117: 1296–1316. DOI: 10.1160/TH16-12-0943 [PubMed: 28569921]
56. Manten A, de Winter RJ, Minnema MC, ten Cate H, Lijmer JG, Adams R, Peters RJ, van Deventer SJ. Procoagulant and proinflammatory activity in acute coronary syndromes. *Cardiovasc Res*. 1998; 40: 389–395. DOI: 10.1016/s0008-6363(98)00130-8 [PubMed: 9893733]
57. McKenzie ME, Pothula A, Gurbel PA, Fuzaylov SY, O'Connor CM, Gattis WA, Serebruany VL. Failure of thrombin generation markers to triage patients presenting with chest pain. *Cardiology*. 1999; 92: 53–58. DOI: 10.1159/000006946 [PubMed: 10640797]
58. Loeffen R, van Oerle R, Leers MP, Kragten JA, Crijns H, Spronk HM, Ten Cate H. Factor Xla and Thrombin Generation Are Elevated in Patients with Acute Coronary Syndrome and Predict Recurrent Cardiovascular Events. *PLoS One*. 2016; 11 e0158355 doi: 10.1371/journal.pone.0158355 [PubMed: 27419389]
59. Ten Cate H, Hemker HC. Thrombin Generation and Atherothrombosis: What Does the Evidence Indicate? *J Am Heart Assoc*. 2016; 5 doi: 10.1161/jaha.116.003553
60. Becker RC, Bovill EG, Corrao JM, Ball SP, Ault K, Mann KG, Tracy RP. Dynamic Nature of Thrombin Generation, Fibrin Formation, and Platelet Activation in Unstable Angina and Non-Q-Wave Myocardial Infarction. *J Thromb Thrombolysis*. 1995; 2: 57–64. DOI: 10.1007/bf01063163 [PubMed: 10639214]
61. Rho R, Tracy RP, Bovill EG, Ball SP, Becker RC. Plasma Markers of Procoagulant Activity Among Individuals with Coronary Artery Disease. *J Thromb Thrombolysis*. 1995; 2: 239–243. DOI: 10.1007/bf01062716 [PubMed: 10608030]
62. van der Bom JG, Bots ML, Haverkate F, Meijer P, Hofman A, Kluft C, Grobbee DE. Activation products of the haemostatic system in coronary, cerebrovascular and peripheral arterial disease. *Thromb Haemost*. 2001; 85: 234–239. [PubMed: 11246539]
63. Schiele F. Fondaparinux and acute coronary syndromes: update on the OASIS 5-6 studies. *Vasc Health Risk Manag*. 2010; 6: 179–187. DOI: 10.2147/vhrm.s6099 [PubMed: 20407625]
64. Reganon E, Vila V, Martinez-Sales V, Vaya A, Aznar J. Inflammation, fibrinogen and thrombin generation in patients with previous myocardial infarction. *Haematologica*. 2002; 87: 740–745. [PubMed: 12091125]
65. Bentzon JF, Otsuka F, Virmani R, Falk E. Mechanisms of plaque formation and rupture. *Circ Res*. 2014; 114: 1852–1866. DOI: 10.1161/CIRCRESAHA.114.302721 [PubMed: 24902970]

Highlights

- The aPL profile of membranes from platelets, leukocytes and EV in patients with ASCVD is characterized in detail using lipidomics, and EV aPL levels were found to be generally higher in ASCVD groups than HC.
- Thrombin generation, EV counts and aPL levels are elevated in EV membranes of patient groups as compared to healthy controls suggesting these particles interact directly with coagulation factors to drive thrombotic risk in ASCVD.
- Membranes from leukocytes were also suggested to contribute somewhat, although platelet aPL did not appear to drive elevated thrombin generation in patients.

Clinical Perspective

What are the clinical implications?

- The EV aPL membrane is proposed as a therapeutic target for dampening thrombotic risk in ASCVD.
- Targeting circulating aPL membrane levels or blocking their interaction with coagulation factors could reduce thrombotic risk in ASCVD.

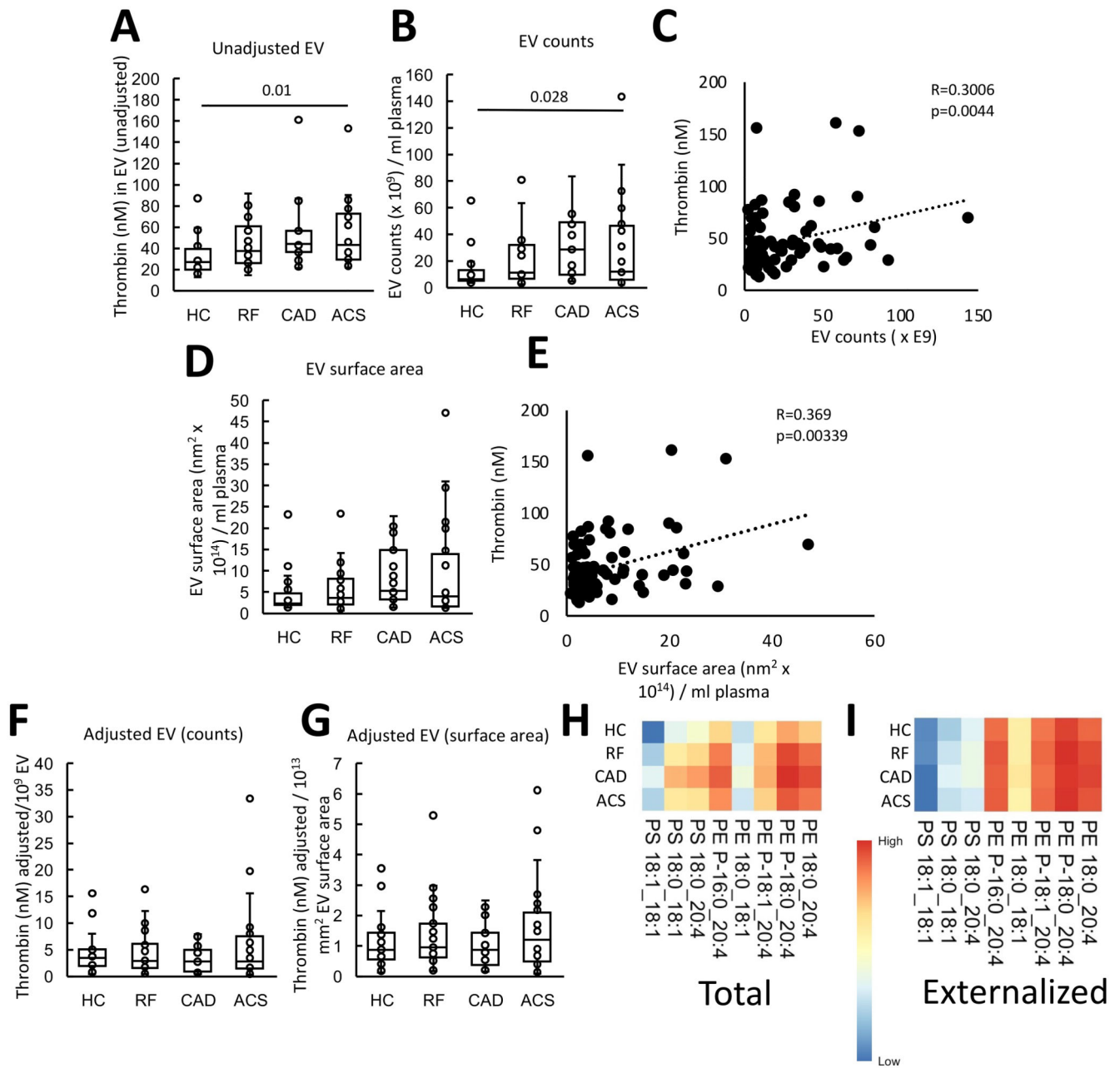


Figure 1. Plasma EV support elevated thrombin generation in ASCVD plasma.

Panel A. EV from patients support higher levels of thrombin generation. The ability of EV membranes to support thrombin generation was assessed using prothrombinase assay as described in Methods. *Panel B. EV counts are significantly higher in patients.* To quantify EV, platelet-free plasma (0.5 mL) was processed using size-exclusion chromatography (iZON qEV columns) and nanoparticle tracking analysis (Nanosight 300). *Panel C. Thrombin generation positively correlates with EV counts.* Thrombin generation and EV counts were correlated using Pearson’s correlation. *Panel D. EV surface area is increased in vascular disease.* EV surface area was calculated as described in Methods and plotted as box

plots. *Panel E. Thrombin generation positively correlates with EV surface area.* Thrombin generation and EV surface area were correlated using Pearson's correlation. *Panel F,G. Thrombin generation was not changed between groups if EV were normalized by counts or surface area.* Thrombin generation on the surface of EV was adjusted by EV counts (per 1×10^9 EV) (Panel F) and surface area (Panel G) and plotted as box plots. *Panels H,I. Heatmaps showing aPL molecular species in EV.* Heatmaps were drawn using the pheatmap R package as described in methods to visualize aPL amounts between groups for all the measured species, analyzed using LC/MS/MS. Statistical significance was tested with one way ANOVA and Tukey Post Hoc test ($p < 0.05$ considered significant). ACS: acute coronary syndrome (n=24 Panel A,B,D,F,G, n=21 panels H,I), CAD: coronary artery disease but no ACS (n=19 panel A,B,D,F,G), RF: Risk factors with no significant coronary artery disease (n=23 panel A,B,D,F,G, n=22 panels H,I), HC: Healthy control (n=24, panels A, n=22 panels B,D,F,G, n=23 panels H,I).

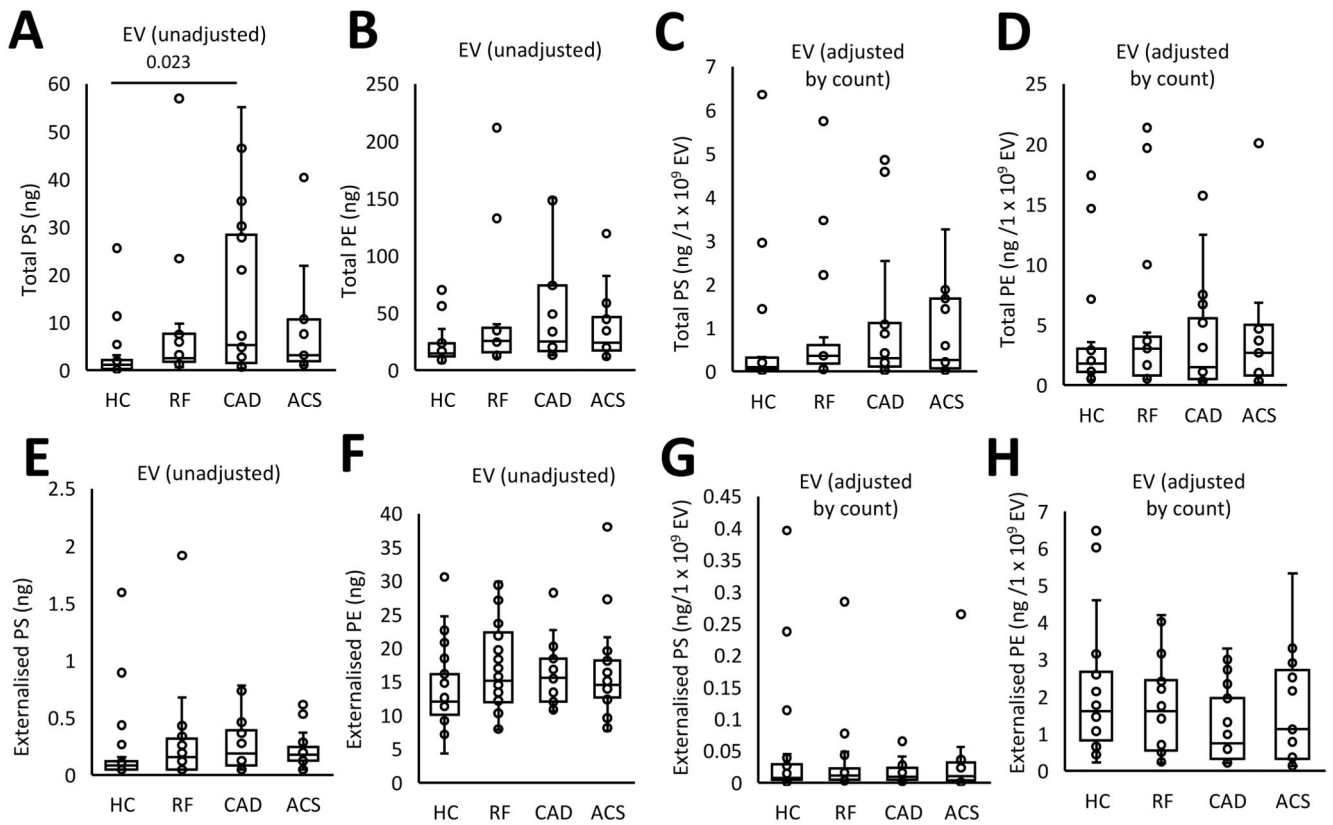


Figure 2. Levels of externalized PE and PS on EV reflect higher EV counts in patients. Panels A,B. Grouping by headgroup, the amounts of total PS and total PE for each of the clinical groups were plotted to examine for differences between groups. Panel C,D. Total aPL amounts adjusted by EV count. Panel E,F. Grouping by headgroup for externalized PS and PE lipids. Panel G,H. Externalized aPL amounts adjusted by EV count. Lipids were extracted from EV-rich plasma fractions as in Materials and Methods. Lipids amounts (ng) were calculated by LC/MS/MS. Statistical significance was tested with one way ANOVA and Tukey Post Hoc test ($p < 0.05$ considered significant). ACS: acute coronary syndrome (n = 19 panels A-D, n=21 panels E-H), CAD: coronary artery disease but no ACS (n = 18 panels A-D, n=19 panels E-H), RF: Risk factors with no significant coronary artery disease (n = 17 panels A,B,C,D, n=22 E,F,G,H), HC: Healthy control (n=23 panels A,B,E,F, n=22 panels C,D,G,H).

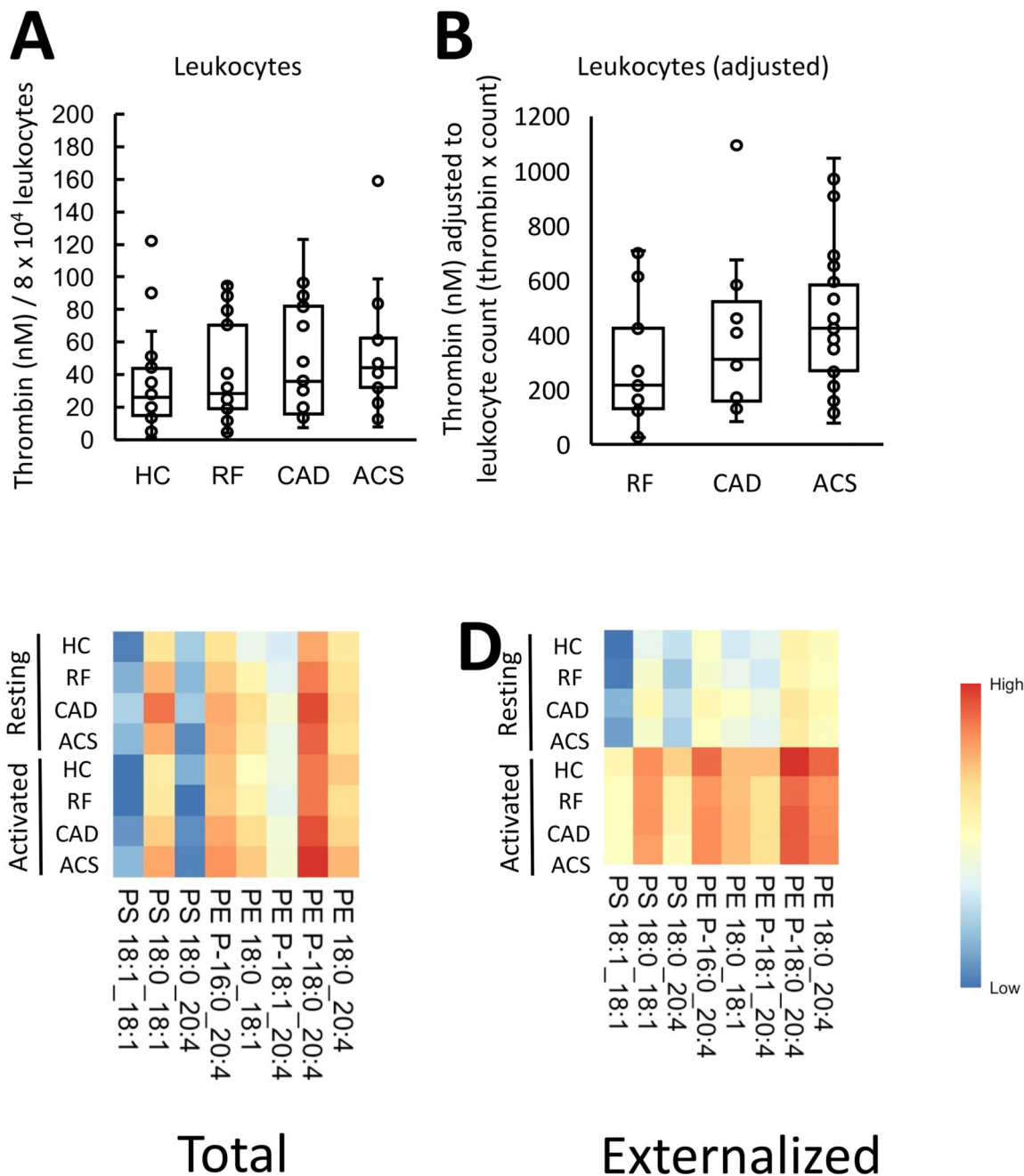


Figure 3. Leukocytes from ACS patients generated more thrombin than HC, and this may be further increased by higher *in-vivo* leukocyte counts.

Panel A. Leukocytes from ACS patients support higher levels of thrombin generation than HC. The ability of leukocyte membranes to support thrombin generation was quantified using prothrombinase assay as described in Methods and displayed on a box plot. Panel B. Adjusting by total leukocyte count demonstrates an upward trend in thrombin generation between RF, CAD and ACS samples. Thrombin generation was adjusted by total leukocyte count to account for differences between groups. Panels C,D. Heatmaps show aPL molecular species in leukocytes, with increased externalization upon activation. Lipids were extracted

from resting or ionophore activated leukocytes and quantified using LC/MS/MS as described in Methods. The log₁₀ lipid amounts (ng) were plotted on a heatmap using the pheatmap R package as described in Methods to show total (Panel C) and externalized (Panel D) aPL molecular species. Statistical significance was tested with one way ANOVA and Tukey Post Hoc test ($p < 0.05$ considered significant). ACS: acute coronary syndrome (n=24, n=21 panels C,D), CAD: coronary artery disease but no ACS (n=19 panel A, n=17 panel B), RF: Risk factors with no significant coronary artery disease (n=23, n=22, panels C,D), HC: Healthy control (n=24 panel A, n=23 panels C,D).

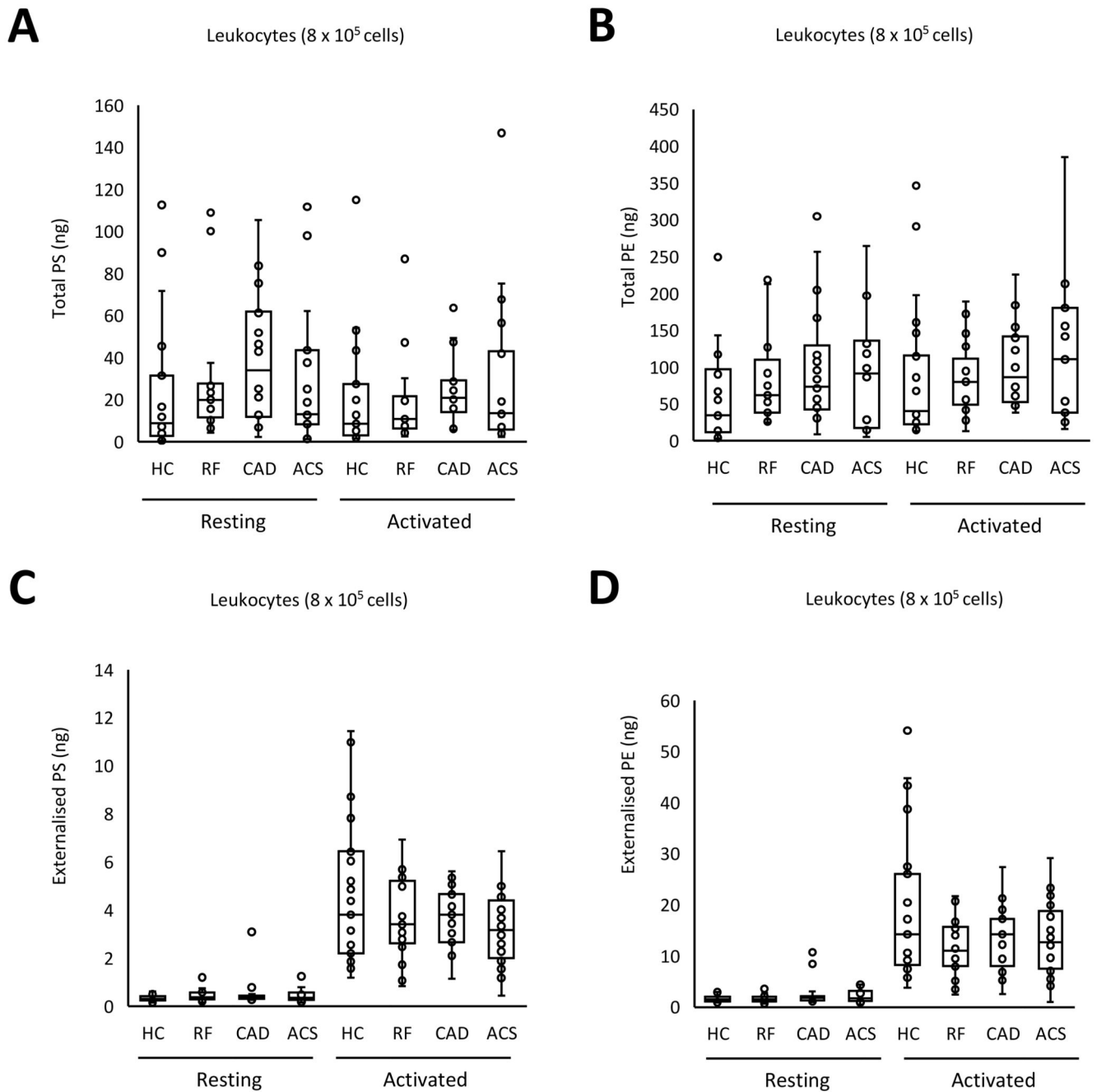


Figure 4. Leukocytes from ACS patients contain and externalize similar amounts of PE/PS, when expressed on a per cell basis.

Panels A-D. Lipids were extracted from resting or ionophore-activated leukocytes as described in Methods. Lipids amounts (ng) were determined using LC/MS/MS. Statistical significance was tested with one way ANOVA and Tukey Post Hoc test ($p < 0.05$ considered significant). ACS: acute coronary syndrome ($n = 19$ panels A,B, $n=21$ panels C,D), CAD: coronary artery disease but no ACS ($n = 18$ panels A,B, $n=19$ panels C,D), RF: Risk factors with no significant coronary artery disease ($n = 17$ panels A,B, $n=22$ panels C,D), HC: Healthy control ($n=23$).

Tukey Post Hoc test ($p < 0.05$ considered significant). ACS: acute coronary syndrome (n=24 panel A, n=21 panels B,C), CAD: coronary artery disease but no ACS (n=19), RF: Risk factors with no significant coronary artery disease (n=23 panel A, n=22 panels B,C), HC: Healthy control (n=24, panel A, n=23 panels B,C).

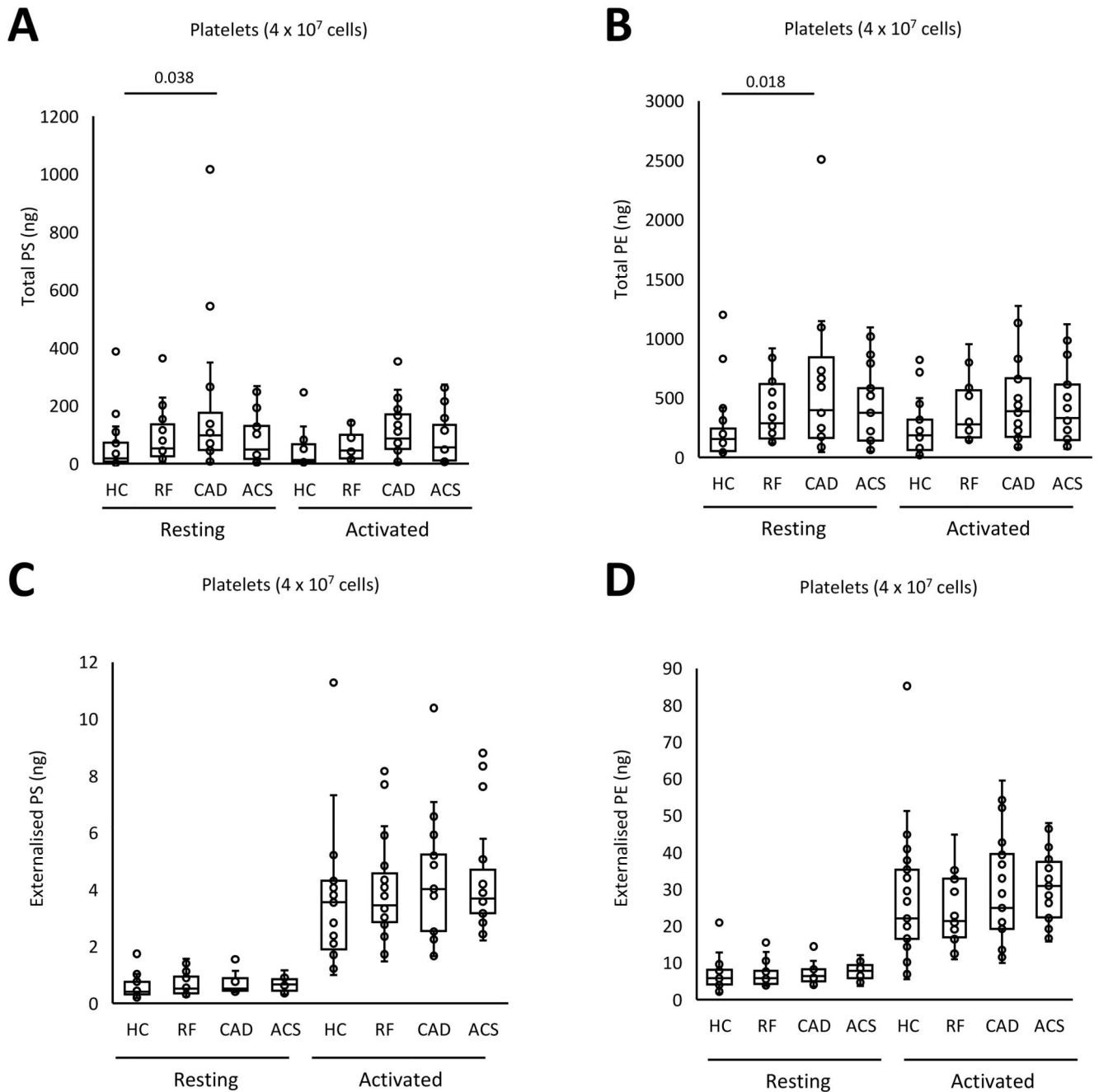


Figure 6. Minimal differences in aPL externalization between groups were seen
Panels A-D: Total and externalized PE and PS for platelets. Lipids were extracted from resting or thrombin-activated platelets as in Methods. Lipids amounts (ng) were determined using LC/MS/MS. Statistical significance was tested with one way ANOVA and Tukey Post Hoc test ($p < 0.05$ considered significant). ACS: acute coronary syndrome ($n = 19$ panels A,B, $n=21$ panels C,D), CAD: coronary artery disease but no ACS ($n = 18$ panels A,B, $n=19$

panels C,D), RF: Risk factors with no significant coronary artery disease (n = 17 panels A,B, n=22 panels C,D), HC: Healthy control (n=23).