Characterisation of platelet phospholipids in unclassified bleeding disorders and deep vein thrombosis

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Thesis submitted to Cardiff University in partial fulfilment of the requirement for the Degree of Doctor of Philosophy (PhD)

2020

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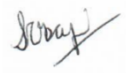
Signed:  Date: 24/03/2020

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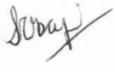
Signed:  Date: 24/03/2020

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Publications

Full papers

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Redpath KA, Aldrovandi MA, Lauder SN, Gketsopoulou A, Tyrrell VJ, Slatter DA, Andrews R, Watkins WJ, Atkinson G, McNeill E, Gilfedder A, Protty M, Burston J, Johnson SRC, Rodrigues PRS, Jones DO, Lee R, Handa A, Channon K, Obaji S, Alvarez-Jarreta J, Kronke G, Ackermann J, Jenkins PV, Collins PW, O'Donnell VB. Phospholipid membranes drive abdominal aortic aneurysm development through stimulating coagulation factor activity. Proceedings of the National Academy of Sciences, 2019. 116(16): p. 8038-8047.

Presentations

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Obaji S, Slatter D, Jenkins PV, O'Donnell VB, Collins P. Characterisation of platelet membrane phospholipids in unclassified bleeding disorders (2018). Infection and Immunity Annual Meeting, Cardiff City Stadium (Poster)

Summary

Patients with a significant bleeding history and normal routine laboratory tests are labelled as having unclassified bleeding disorder (UBD). Approximately one third of patients with acute deep vein thrombosis (DVT) have no risk factor identified and are labelled idiopathic. The experiments conducted herein investigate whether the phospholipid composition of the platelet membrane is contributory to the clinical phenotype.

The ability of platelets and microvesicles to support thrombin generation was investigated using a thrombin generation assay tailored to be sensitive to the phospholipid membrane. Peak thrombin generation supported by washed platelets and microvesicles was reduced in UBD patients compared with healthy controls. Peak thrombin and velocity index were increased in patients with DVT.

To determine whether changes in thrombin generation could be attributed to native aminophospholipids in the platelet membrane, Phosphatidylserine (PS) and Phosphatidylethanolamine (PE) were measured by mass spectrometry following thrombin-activation of platelets. The thrombin generation assays were sensitive to externalised PE/PS as demonstrated by the minimal amount of thrombin generated by the platelets of a Scott syndrome patient. Externalised PE/PS species were similar in disease cohorts and healthy controls.

Previous studies demonstrate that enzymatically oxidised phospholipids produced rapidly on platelet activation support thrombin generation in vitro. Overall, trends of lower quantities of procoagulant 12-HETE-PE and 12-HETE-PC species were measured in UBDs compared with healthy controls whereas 12-HETE-PE species were higher in DVT patients. Lastly, in UBD patients receiving desmopressin for invasive procedures there was increased externalisation of PS and a trend towards increased peak thrombin generation supported by washed platelets.

The observed changes in procoagulant oxidised phospholipids suggest the phospholipid composition of the platelet membrane may be implicated in haemostatic disorders. Desmopressin may be effective in UBD because it increases externalised PS and supports thrombin generation. Further studies are required to confirm these findings.

Abbreviations

AA	Arachidonic acid
ACD	Acid-citrate-dextrose acid
a.m.u.	atomic mass units
ATP	Adenosine 5'-triphosphate
Ca^{2+}	Calcium
CAT	Calibrated automated thrombography
CoA	Coenzyme A
COX	Cyclooxygenase
cps	counts per second
DDAVP	Desmopressin
DMPE	Dimyristoyl phosphatidylethanolamine
DMSO	Dimethyl sulfoxide
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DVT	Deep vein thrombosis
eoxPL	Enzymatically oxidised phospholipid
HETE	Hydroxyeicosatetraenoic acid
HPLC	High pressure liquid chromatography
IP3	Inositol 1,4,5-triphosphate
LC	Liquid chromatography
LOX	Lipoxygenase
LPCATs	Lysophosphatidlycholine acyltransferase
MBOAT	Membrane-bound-O-acetyltransferases
MS	Mass spectrometry
m/z	Mass to charge ratio
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
ng	nanograms
OxPL	Oxidised phospholipid

PAR	Protease-activated receptor
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
pg	Picograms
PG	Prostaglandin
PI	Phosphatidlyinositol
PISKs	Phosphatidlyinositide 3-kinases
PIP2	Phosphatidlyinositol 4,5-bisphosphate
PKC	Protein kinase C
PL	Phospholipid
PLA2	Phospholipase A2
PLC	Phospholipase C
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
SAPE	1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine
SAPS	1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-L- serine
TG	Thrombin generation
TF	Tissue factor
TXA	Tranexamic acid
TXA2	Thromboxane A2
TXB2	Thromboaxane B2
UBD	Unclassified bleeding disorder
VWF	Von Willebrand Factor
x g	Multiples of gravity

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Chapter 1 - General Introduction

1.1. Normal haemostasis

Haemostasis is a rapid and localised process involving a tightly regulated balance of procoagulant and anticoagulant mechanisms. Excessive bleeding or unwanted thrombosis occur when certain elements involved in these processes are dysfunctional or absent. Common manifestations of clotting dysregulation in the clinical setting include venous thromboembolism and acute coronary syndrome or abnormal bleeding following a haemostatic challenge such as surgery.

The combined processes of haemostasis result in the production of a haemostatic plug and the formation of an occlusive thrombus at the site of vascular injury. Platelets are essential for maintaining normal haemostasis. The initial interaction of circulating platelets with the disrupted vessel wall mediates the formation of a platelet plug at the injury site in a process referred to as primary haemostasis. Additionally, the platelet surface is critical for the control of coagulation reactions that lead to thrombin generation and fibrin clot formation called secondary haemostasis [1]. To date, studies investigating the role of platelets in the pathophysiology of haemorrhagic or thrombotic disorders have focused mainly on platelet number and tests of platelet aggregation [2-6]. This investigative approach has identified many disorders associated with abnormal bleeding. For example, inherited defects in platelet aggregation or adhesion such as Glanzmann's thrombasthenia and Bernard Soulier syndrome are associated with moderate to severe haemorrhage. A deficiency in platelet number or function may be associated with haemostatic failure in acquired conditions such as liver and renal disease. Thrombocytopenia encountered in the clinical setting has many underlying causes including bone marrow failure and immune mediated as well as inherited pathologies. In some hereditary platelet and bleeding disorders, the cause of impaired haemostasis has remained undefined with current available coagulation assays and platelet function tests and these are usually termed unclassified bleeding disorders. In recent years, large scale projects including the BRIDGE-BPD study have focused on the use of gene panels and genome sequencing in order to identify causal gene variants associated with uncharacterised platelet and bleeding disorders [7]. This approach has led to the discovery of novel mutations [8, 9] and has increased the number of genes implicated in bleeding and platelet disorders to a total of 76 [10]. However, a large proportion of cases of unclassified bleeding disorders in this study had no identifiable mechanism to account for the clinical bleeding phenotype.

Whilst it is well known that platelets are important for providing a membrane surface for the interaction of coagulation factors, the contribution of the platelet procoagulant membrane in the pathophysiology of coagulation disorders is an unexplored area. In this study, the role of the platelet membrane is investigated in both unexplained bleeding and venous thrombosis.

1.2 Platelets

Platelets are small, cell fragments without a nucleus typically measuring 2-3µm in diameter. They are derived from megakaryocytes which are large haematopoietic cells sparsely distributed in the bone marrow [11]. Following DNA expansion during maturation, megakaryocytes enlarge and develop thick cytoplasmic pseudopods leading to the formation of pro-platelets. The pro-platelets are shed into bone marrow sinusoids in a process of cytoplasmic fragmentation. Individual platelets are subsequently released from the end of the pro-platelet [12, 13]. This

process is regulated by thrombopoietin (TPO) and its cell receptor c-mpl [14]. It is estimated that about 3000 platelets are generated from each megakaryocyte [15]. Platelets have a life-span of 7-10 days, and circulate with a normal count of 150-400 x 10^9/L in humans [16]. They are ultimately sequestered in the spleen and by Kupffer cells in the liver [17].

In the resting state, platelets have a disc-like shape due to a complex cytoskeleton composed of spectrin, actin filaments and microtubules [18]. Despite the absence of a nucleus, platelets conserve messenger RNA (mRNA) transferred from the megakaryocyte and are able to synthesise proteins within their endoplasmic reticulum [12]. The main organelles include lysosomes, mitochondria and α and dense storage granules, both of which are released upon platelet activation [12]. The more abundant α granules (40-80 per platelet) contain proteins involved in platelet adhesion and aggregation (e.g. VWF, fibrinogen) as well as several membrane integrins (e.g. GPIIbIIIa, P-selectin) [19]. Other constituents include growth factors and chemokines which play a role in vessel wall repair, inflammation and host defence. Dense granules (3-8 per platelet) contain ATP, ADP, serotonin (5HT), calcium and polyphosphates [20, 21].

The platelet surface membrane contains five types of integrin receptors, each composed of α and β subunit, which mediate adhesive interactions and aggregation [22] [23, 24]. Following exposure to specific agonists, transmembrane G-protein coupled receptors (GCPR) initiate the amplification stage of platelet activation through intracellular signalling pathways. The main soluble agonists and their membrane receptors include thrombin (PAR1, PAR4), ADP (P2Y1, P2Y12), epinephrine (α2-adrenergic receptors) and thromboxane A2 (TPα and β) [25].

The open canicular system (OCS) is a widespread network of channels which extends into invaginations of the platelet membrane. This serves to increase the surface area of the membrane during platelet spreading and facilitates the release of storage granule contents [26]. Within the cytoplasm, a closed channel network known as the dense tubular system (DTS) forms part of the endoplasmic reticulum where calcium is stored and released on activation [27].

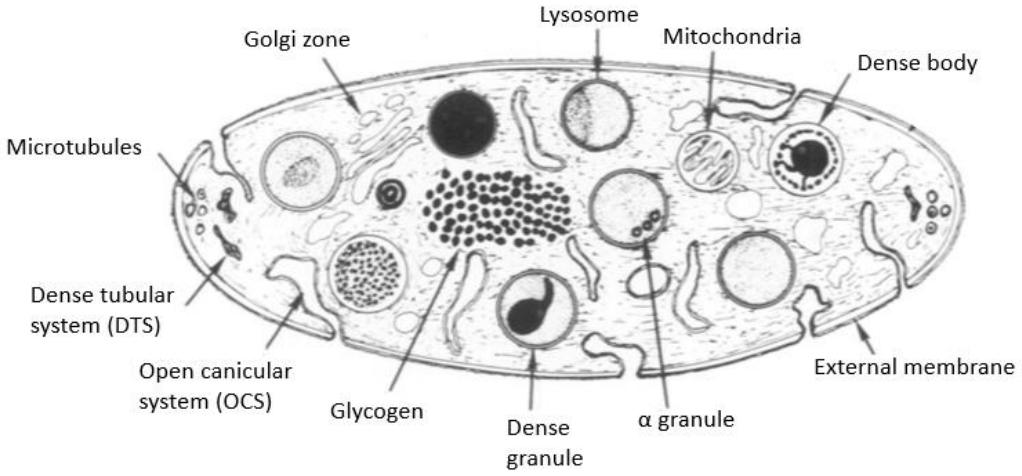


Figure 1.1 Ultrastructure of a platelet (cross-sectional plane). The surface membrane extends into channels of the open canalicular system (OCS), which provides a pathway for the transport of granule contents. The cytoskeleton is maintained by circumferential microtubules and the organelles are contained within the cytosol. Used with permission from White JG, Gerrard JM. Ultrastructural features of abnormal platelets. A review. The American Journal of Pathology, 1976. 83: 590 [28]

1.3 Haemostasis

1.3.1 Primary haemostasis

Platelets play a central role in the generation of a haemostatic plug which prevents blood loss at the site of vessel injury. The undisrupted vascular endothelium releases metabolites such as nitric oxide (NO) and prostaglandin (PGI2) which promote vasodilation but also have inhibitory actions on circulating platelets[29]. Following injury to the vessel wall, the initial step in haemostasis involves the tethering of platelets to the site of injury which occurs through the interaction of platelet complex GPIb-V-IX and extravascular VWF bound to collagen. In addition, other subendothelium matrix proteins including fibronectin and laminin are exposed which enable the platelets to adhere [30] [31]. The damaged endothelium responds by exhibiting vasoconstriction through secretion of mediators such as endothelin-1 (ET-1), which also induces smooth muscle growth and the synthesis of growth factors such as platelet-derived growth factor (PDGF) [32].

VWF is a large multimeric glycoprotein which is produced in vascular endothelial cells and megakaryocytes. It is stored in endothelial Weibel-Palade bodies prior to release whilst some undergoes abluminal secretion from the endothelial cell and thus is directly exposed to subendothelial collagen [33, 34]. Plasma VWF is mainly derived from endothelial cells, although a small proportion is released from the α granules of activated platelets [35]. Above a shear rate of 500-800/sec, VWF undergoes a structural change which facilitates interactions with platelet GPIb and collagen via its A1 and A3 domain respectively [36].

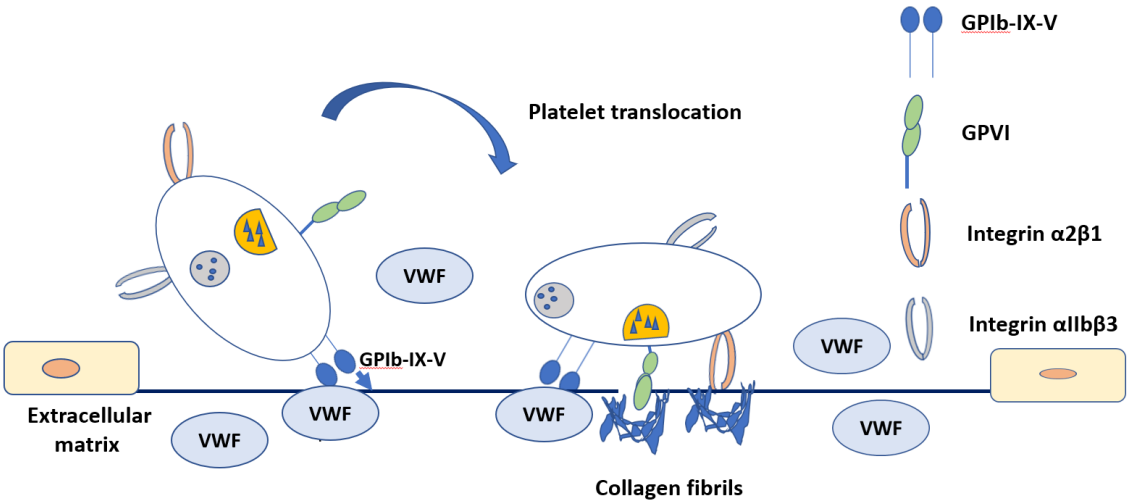


Figure 1.2 Schematic diagram of initial platelet-vessel wall interaction. Circulating platelets contact exposed components of the extracellular matrix at the site of vascular injury, and slow down sufficiently to adhere through the binding of GPIb-V-IX to VWF deposited on the subendothelium. Stable adhesion of the rolling platelet involves the interaction of platelet integrins with their ligands. Both the GPVI receptor and integrin α2β1 bind collagen.

The ligand action of VWF enables platelets at the vessel wall to bind collagen via the GPVI receptor so that a firm adherence is formed. GPVI, the main collagen receptor belongs to the immunoglobulin superfamily and has two IgG domains with an associated FcR adaptor protein (FcRϒ). The binding of collagen to FcRϒ initiates a tyrosine kinase pathway characterised by activation of phospholipase Cγ2 (PLCγ2) [37, 38]. This leads to downstream signalling events which involve the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphonate (PIP2) to form diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 binds to specific receptors of the dense tubular system to initiate calcium mobilisation, which triggers the release of platelet storage granule contents (e.g. ADP, thromboxane A2, adhesion molecules)[39, 40] [41]. Through secondary mechanisms, platelet receptors α2β1 and αIIbβ3 are converted to a high-affinity state which promotes their respective binding to collagen and fibrinogen [42]. Both αIIbβ3 and VWF via its C1 domain can bind fibrinogen which is required for the aggregation of platelets. It is reported that up to 80,000 copies of the αIIbβ3 integrin are present on the platelet surface [43].

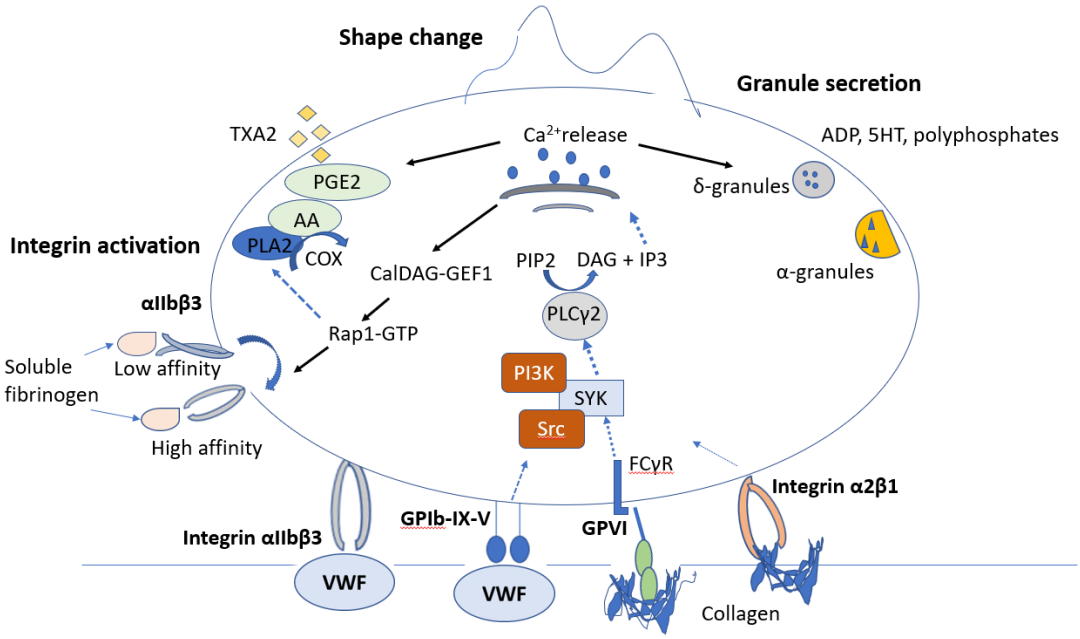


Figure 1.3. Signalling events following platelet adhesion. The binding of collagen to GPVI leads to platelet activation through a tyrosine kinase signalling pathway that involves the kinases Syk and phospholipase Cγ2 (PLC γ2) [37]. PLCγ2 hydrolyses phosphatidylinositol 4,5-bisphosphonate (PIP2) to form secondary messengers diacylglcerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 triggers calcium release from storage in the dense tubular system which leads to granule release, synthesis of thromboxane (TXA2) and shape change. Calcium and DAG-related guanine nucleotide exchange factor 1 (CalDAG-GEF1), a member of the Ras GRP family is a signalling molecule which triggers integrin activation and enhances collagen-dependent TXA production [44, 45]. Image adapted from Mackman et al and Mosawy [46, 47]

The activation of αIIbβ3 involves a conformational change, termed 'inside-out signalling' which increases the affinity of the integrin for fibrinogen [43]. This mechanism is triggered by the soluble agonists ADP, thromboxane A2 and thrombin in addition to collagen [48]. The platelet agonists mediate their effects via activation of G-protein coupled receptors. Through G protein mediated signalling, thromboxane A2, thrombin and ADP induce platelet shape-change which involves polymerisation of the actin-based cytoskeleton, loss of discoid shape and the development of filopodia to promote platelet spreading. These contractile actions are regulated by two main pathways. Firstly, Gq signalling activates phospholipase β (PLC β), and via IP3 formation mobilises calcium from intracellular stores. Calcium is required for myosin light chain kinase (MLCK) activity which induces the phosphorylation of myosin light chain required for the contraction of actin filaments. Secondly, G12/13 signalling activates the Rho/Rho-kinase pathway which is involved in regulating myosin light chain phosphorylation [25, 49]. Additionally, the platelet agonists act as positive feedback regulators by promoting their own production and release thus amplifying the activation process which leads to recruitment of additional platelets in order to increase thrombus growth [25].

Specifically, ADP acts on P2Y1 and P2Y12 receptors [25]. Thromboxane A2, is a short-lived platelet agonist which is synthesised from arachidonic acid stored in the plasma membrane. It exerts its action via the TPα receptor, although both TPα and TPβ isoforms are expressed on platelets [50]. Following hydrolysis by cytosolic phospholipase A2, arachidonic acid is metabolised by cyclo-oxygenase 1 (COX-1) and thromboxane synthase to generate the end-product. Thrombin generated from secondary haemostasis is the most potent of the agonists and activates platelets via PAR1 and PAR4 [25].

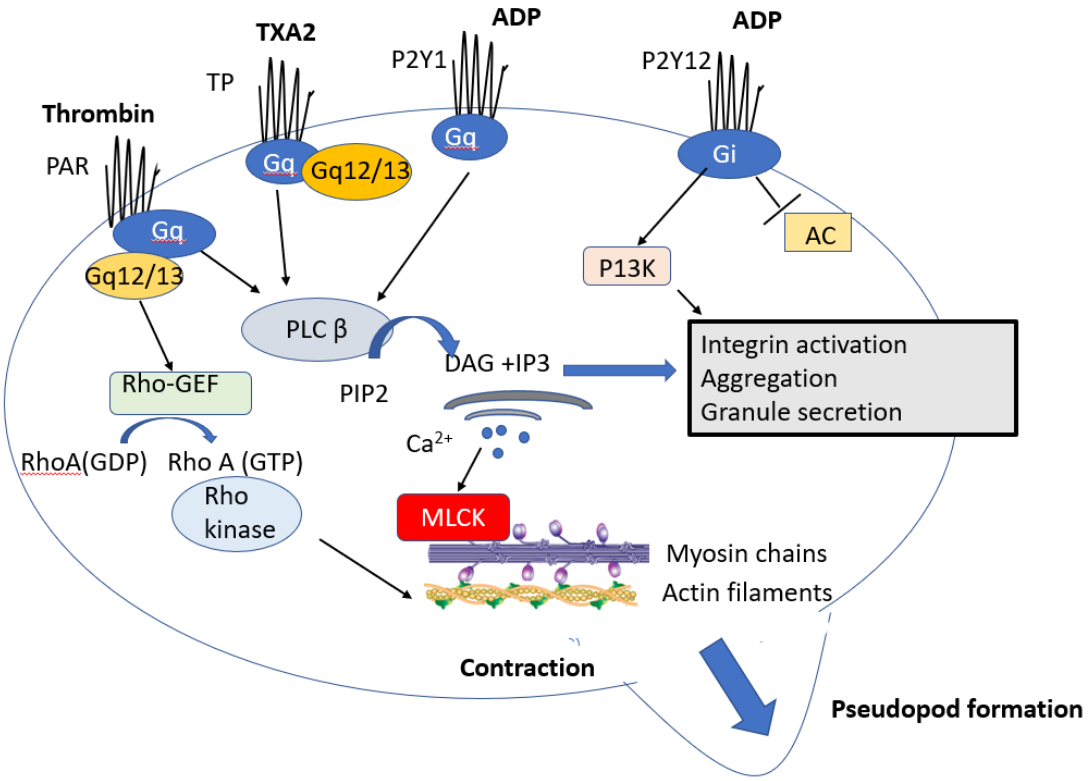


Figure 1.4 Platelet agonists and G protein signalling. ADP, thromboxane (TXA2) and thrombin mediate platelet shape change through Gq signalling which activates phospholipase C (PLC β) and results in calcium mobilisation. Calcium regulates myosin light chain kinase (MLCK) activity which induces the phosphorylation of myosin light chain required for platelet contraction. Thrombin and thromboxane also mediate Gq12/13 signalling which activates the Rho/Rho kinase pathway which regulates actin-myosin interactions. P2Y12 linked to Gi inhibits adenyl cyclase (AC) enhancing platelet aggregation and additionally activates PI3K. Figure adapted from Stegner et al [49]

A sub-population of platelets dually activated by collagen and thrombin - (COAT) platelets – expose serotonin (5HT) and retain several α granule proteins on their negative phospholipid surface and are considered the most procoagulant forms within the platelet plug [51]. Other studies report the additional clustering of coagulation factors on the surface of COAT platelets, thus forming a procoagulant "cap" [52]. Polyphosphate (PolyP) released from the dense granules during platelet activation is recognised to have an important role in promoting coagulation through its interaction with nearby coagulation factors. Short-chain polymers of polyP facilitate the activation of Factor V and Factor XI by acting as a co-factor for thrombin. Long-chain polyphosphates more so than their shorter counterparts, bind the fibrin clot subsequently formed thereby increasing its stability [53, 54].

1.3.2. Secondary haemostasis

This procoagulant system is initiated when tissue factor (TF) is exposed following vascular injury [55]. TF is a 47-kDa integral transmembrane protein [56] which is expressed by vessel wall cells such as smooth muscle cells and fibroblasts where they have been described as forming a 'haemostatic envelope'or barrier [57]. It is also expressed by tumour cells, foam cells in atherosclerotic plaques and peripheral blood monocytes in response to inflammation [58, 59]. Currently, it is proposed that TF exists in a 'cryptic' or inactive state. The interaction of TF with anionic phospholipids, specifically phosphatidylserine (PS) is recognised to be of importance in the 'decryption' or activation of TF, although the precise mechanisms for decryption is still under debate [60].

Historically, the system initiated by the exposure of FVII to cells expressing TF following vascular injury has been referred to as the extrinsic pathway. The binding of TF to the serine protease Factor VIIa triggers the enzymatic conversion of a cascade of coagulation factors (zymogens) into their active serine forms. Plasma FVIIa, despite circulating at low concentrations (0.005 μg/ml) can form a potent enzymatic complex with TF (TF-VIIa). Alternatively, the more abundant zymogen FVII can be converted to FVIIa either by 'auto-activation' or by downstream serine proteases in a process significantly accelerated by TF [61, 62]. The TF-VIIa complex converts Factor IX to FIXa and Factor X to Xa. The initiation stage produces a trace amount of thrombin which is sufficient to activate Factor XI and cofactors Factor VIIIa and Factor Va. In the amplification phase, Factor XIa cleaves Factor IX to IXa. The newly generated Factor IXa forms a complex with VIIIa and subsequently activates Factor X which binds to FVa. The tenase (FVIIIa-FIXa) and prothrombinase (FXa-FVa) complexes are both dependent on an influx of calcium ions in platelets leading to exposure of anionic phospholipid surface provided by the activated platelet for catalytic efficiency. The propagation phase culminates in the rapid conversion of prothrombin to thrombin by the prothrombinase complex[55, 62] [63]. Thrombin cleaves fibrinogen to fibrin and activates Factor XIII to enable cross-linkage of the insoluble fibrin clot.

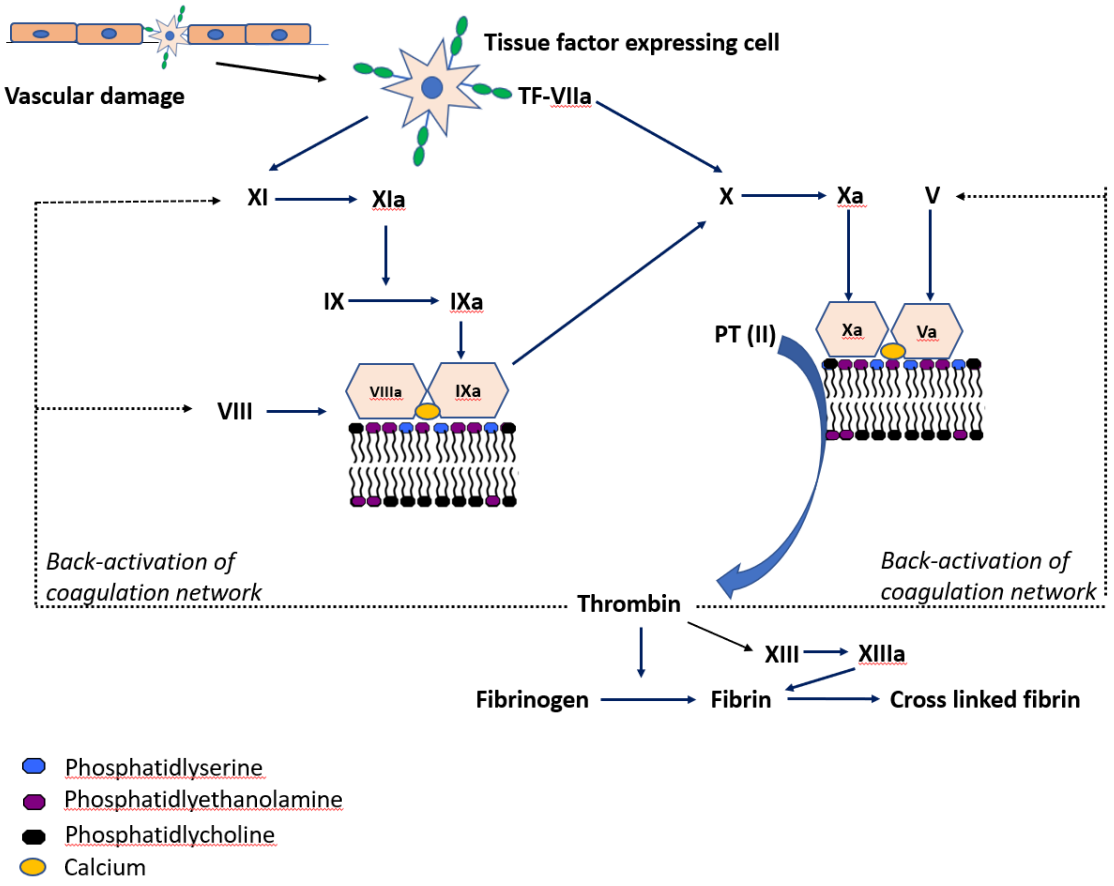


Figure 1.5 Tissue factor driven haemostasis network. Following vessel wall injury, FVII is exposed to tissue factor (TF) -expressing cells. TF-FVIIa complex activates a sequential series of coagulation reactions which initially yields a minimal amount of thrombin. Thrombin back-activates FXI and co-factors FVIII and FV. The tenase (FVIIIa-IXa) and prothrombinase (Xa-Va) complexes assemble in the presence of calcium and membrane phospholipid. This culminates in the 'thrombin burst' that leads to production of a fibrin clot.

The contact or intrinsic pathway is triggered when Factor XII undergoes spontaneous activation upon contact with an artificial surface such as glass or ellagic acid and historically was considered of relevance to haemostasis occurring in vitro. The generation of FXIIa converts prekallikrein into kallikrein and Factor XI into FXIa. This interaction is mediated by the cofactor high molecular weight kininogen (HMWK). FXIa then propagates the formation of the tenase complex with subsequent downstream events. Whilst a natural deficiency of FXII, HMWK or kallikrein does not result in a bleeding disorder, contact activation in the clinical setting is associated with the use of cardiopulmonary bypass circuits and extracorporeal membrane oxygenation [63] [64]. In murine studies, FXII deficiency has been associated with protection against thrombus formation. In a thromboembolism model induced by ADP and collagen, defective thrombus formation was observed in FXII-deficient mice at the site of arterial injury [65]. Similarly, mice lacking FXII were reported to be protected from thrombosis in ischaemic brain injury [66]. It is widely recognised that polyphosphates secreted from activated platelets can directly trigger FXII activation [67]. Other components of coagulation that have been reported as contact pathway activators include PS-liposomes and matrix proteins such as collagen although their roles in this regard are not well defined[68, 69] [70].

The haemostatic process is regulated by the action of several natural anticoagulants. The initial TF-VIIa complex is inhibited by tissue factor pathway inhibitor (TFPI), a serine protease inhibitor composed of three kunitz domains (K1-3). Kunitz domains 1 and 2 binds FVIIa and FXa respectively. Kunitz domain 3 binds Protein S, which promotes the interaction of TFPI with FXa. Thus, TFPI is critical for the inactivation of the extrinsic tenase complex [71]. Factor Xa can also be inhibited by Protein Z-dependent protease inhibitor, requiring co-factor Protein Z [72].

The protein C pathway is another important mechanism for inhibiting the coagulation response to injury and is initiated by the binding of thrombin to thrombomodulin on endothelial cells. Protein C, a vitamin K- dependent zymogen, is activated by the thrombin-thrombomodulin complex and this interaction is enhanced by endothelial protein C receptors (EPCR). Once formed, activated PC together with co-factor Protein S proteolytically cleaves and inactivates factors Va and VIIIa [73, 74].

Finally, the inactivation of thrombin and Factor IXa, Xa and XIa is mediated by antithrombin, a 58kDa serine protease inhibitor produced in the liver [75]. The active serine site of the coagulation protease cleaves the arginine-serine bond in the active region of antithrombin which induces a conformational change in antithrombin resulting in inactivation of the serine protease [76, 77]. Vessel wall glycosaminoglycans such as heparan sulphate or the anticoagulant heparin, significantly increase the rate of inactivation (>1000 fold) [78]. Other inhibitors of thrombin include heparin co-factor II and α2-macroglobulin [79].

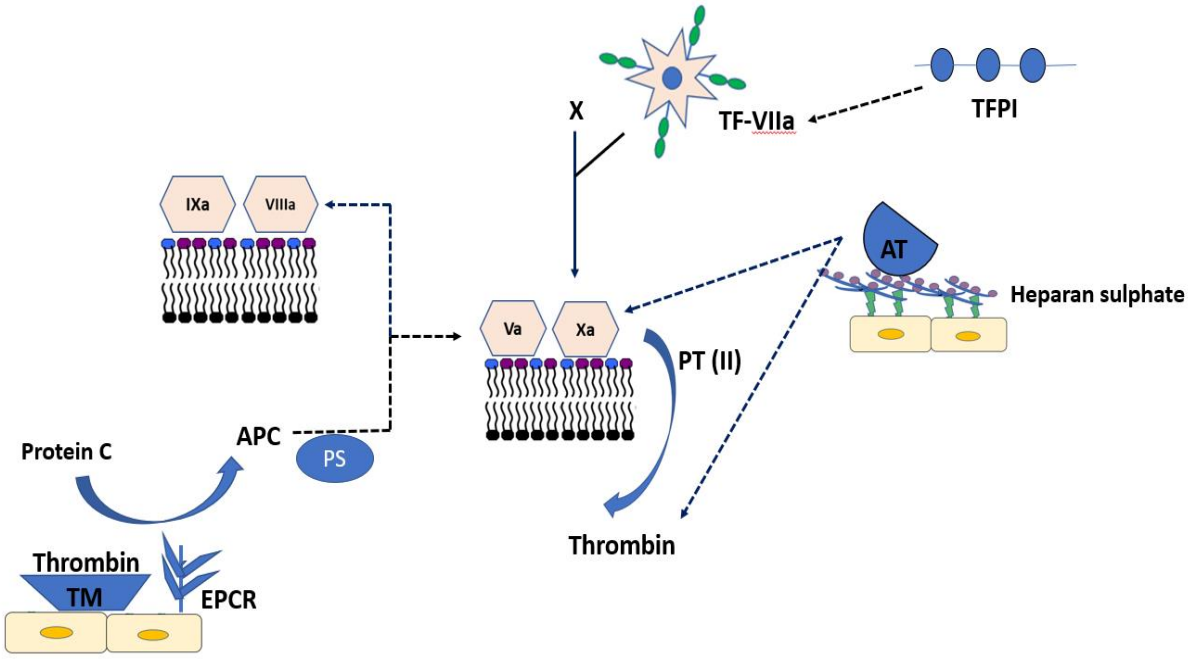


Figure 1.6 Regulators of the haemostasis network. Tissue factor pathway inhibitor (TFPI) inhibits the activity of the TF-VIIa complex. Antithrombin (AT) inhibits thrombin and Xa in addition to serine proteases IXa and XIa. Thrombin bound to thrombomodulin (TM) on the endothelium surface activates Protein C associated with its receptor EPCR. Activated Protein C (APC) and its co-factor Protein S inhibit FVa and FVIIIa.

1.3.3 Fibrinolysis

Fibrinolysis involves the break-down of the fibrin clot formed following injury to a blood vessel. A key component of the pathway is the glycoprotein zymogen plasminogen, the precursor of the active serine protease, plasmin. The conversion of plasminogen to plasmin occurs through cleavage of a single peptide bond (Arg561-Val562) forming Glu-plasmin, which undergoes auto-catalytic degradation to the more active Lys-plasmin. The main plasminogen activator is t-PA which is synthesised and secreted from vascular endothelial cells [80] [81].

Briefly, plasmin degrades the clot by cleaving fibrinogen and fibrin into small, soluble fragments referred to as fibrin degradation peptides (FDP's). The cross-linking of fibrin by thrombin-activated factor XIII, however results in a different fragmentation pattern after plasmin-induced cleavage due to transamidation of the fibrin chains induced by Factor XIIIa [82, 83]. One of the characteristic fragments (D-dimer) can be measured in blood and is of clinical use in determining the likelihood of venous thromboembolism.

Each fibrinolytic step is inhibited by a specific protease. PAI-1 is the main inhibitor of tPA whilst plasmin is inhibited by α-2 antiplasmin. Thrombin activatable fibrinolysis inhibitor (TAFI), an enzyme derived from carboxypeptidase B removes the C-terminal lysines from fibrin that has been partially lysed by plasmin, thereby preventing the binding of plasminogen and tPA. The activation of TAFI occurs following the interaction of thrombin with thrombomodulin [84].

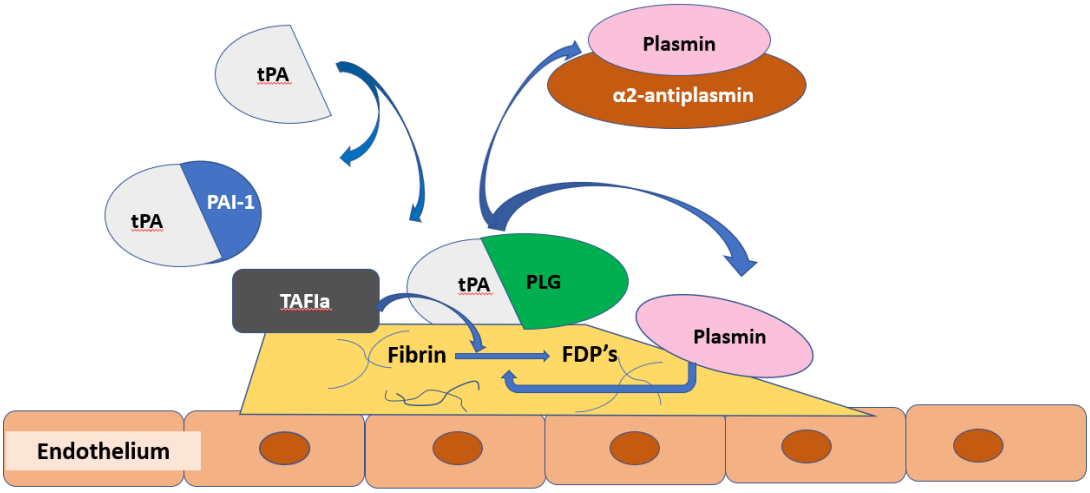


Figure 1.7 The fibrinolytic system. Plasminogen (PLG) and tPA bind to the fibrin clot. The plasminogen is cleaved by tPA to plasmin which then converts fibrin into soluble fibrin degradation products (FDP's). PAI-1 inhibits tPA and α2-antiplasmin inhibits plasmin. TAFIa removes C terminal lysines from partially degraded fibrin thereby preventing the binding of plasminogen and tPA. Adapted from http://themedicalbiochemistrypage.org/blood-coagulation.html.

1.4 Phospholipids

1.4.1. Platelet phospholipid structure

The 'fluid mosaic model' describes the structure of the plasma membrane as a 'mosaic' of membrane proteins embedded in a fluid lipid-rich phase [85]. Phospholipids are the main constituents of the membrane and due to their amphipathic nature, are organised in a bilayer with the polar heads facing outwardly and the nonpolar fatty acid tails orientated inwardly thus forming a hydrophobic barrier. Other membrane components include sphingomyelin, cholesterol and transmembrane glycoproteins [86]. Diacyl phospholipids are a common subclass of lipids within the membrane and are composed of two hydrophobic fatty acids which are each attached to the Sn1 and Sn2 positions of a glycerol backbone by an acyl bond. The polar phosphate-containing headgroup is esterified to the Sn3 position [87]. Their classification is based on the nature of the head-group and the length and saturation of the fatty acid chains. Whilst the head-group dictates the charge of the membrane, the fatty acid chains influence the curvature, thickness and fluidity [88-90].

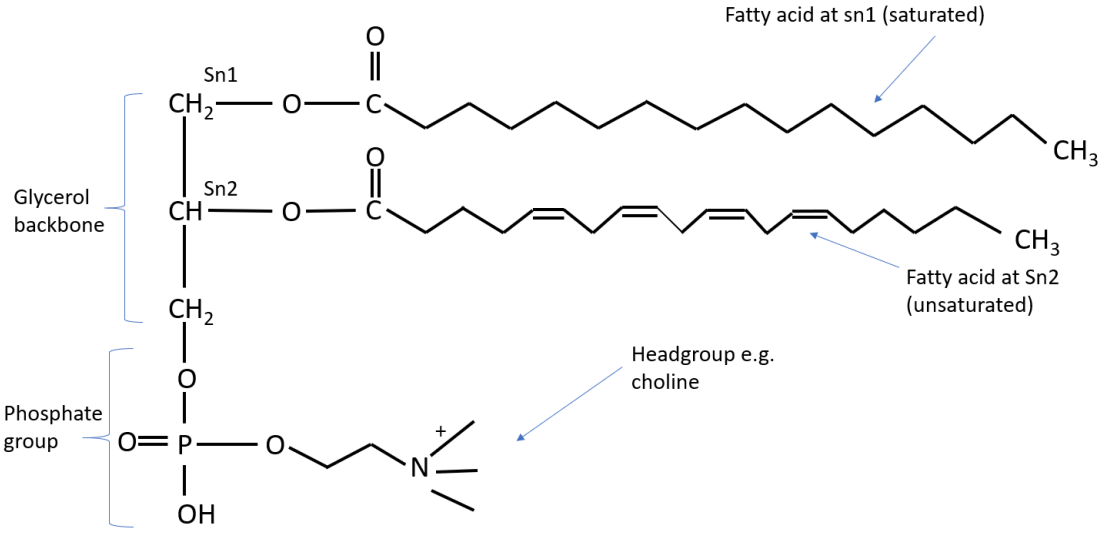


Figure 1.8. Example of a diacyl phospholipid. The structural components include the glycerol backbone, the phosphate group and the organic head group. Fatty acid chains occupy the Sn1 and Sn2 position of the glycerol backbone. The presence of a double bond indicates an unsaturated fatty acid chain. Adapted from Hanna et al[91]

In platelets, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant species accounting for 35-38% and 27-35% of total phospholipids respectively. Phosphatidlyserine (PS) contributes approximately 10-15% and phosphatidylinositol (PI) is the least abundant representing only 5% of the total [92]. The fatty acid chains are diverse with regards to their carbon chain length and degree of saturation, referring to the presence of carbon double bonds (unsaturated) or their absence (saturated) [90]. The Sn2 position is generally occupied by polyunsaturated fatty acids whilst saturated fatty acids occupy the Sn1 position [93]. Arachidonic acid (20:4, 20 carbon and 4 double bonds) is one of the most abundant fatty acid in platelet phospholipids. Others include oleic acid (18:1), linoleic acid (18:2), stearic acid (18:0) and palmitic acid (16:0) [94]. Recently, the composition of the fatty acid chain has been reported to influence coagulation with longer unsaturated fatty acid chains supporting thrombin generation somewhat better than shorter fatty acid chains [95].

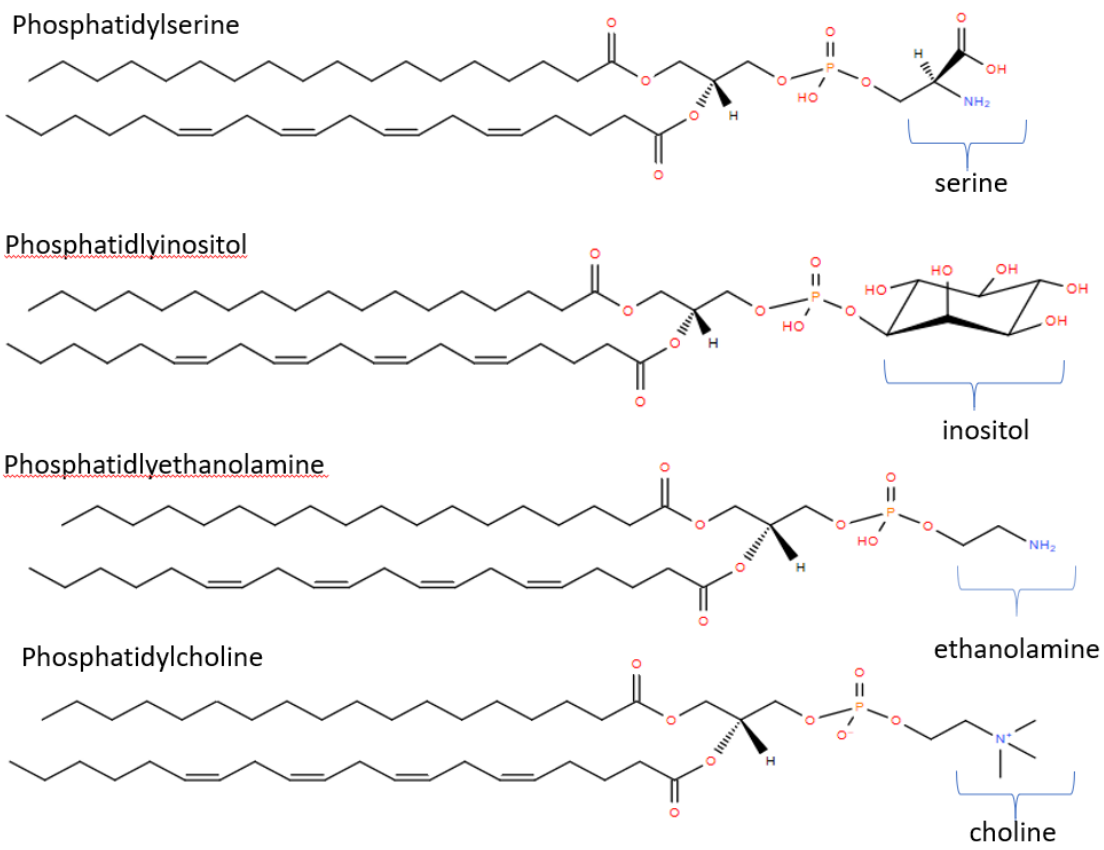


Figure 1.9 Structure of the main diacyl phospholipid species. The 4 main species of phospholipid in the platelet membrane is shown. Images constructed using LIPIDMAPS resources. www.lipidmaps.org/resources/tools/index.php

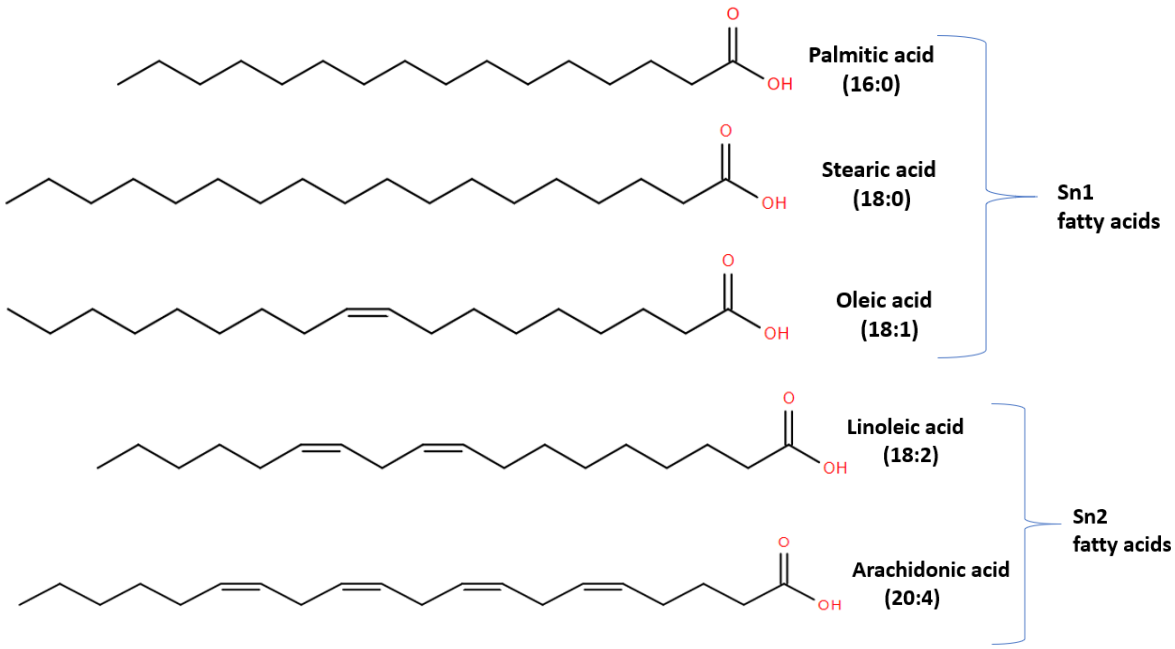


Figure 1.10 Structures of the more common Sn1 fatty acids and Sn2 fatty acids in platelets. The first number within the bracket denotes the number of carbon atoms and the second number indicates the total number of double bonds in the fatty acid. Images constructed using LIPIDMAPS resources. www.lipidmaps.org/resources/tools/index.php

More structural variety is introduced by the type of bond (vinyl ether or acyl) present at the Sn1 position of the glycerol backbone. The plasmalogen subclass of phospholipids has a characteristic structure where the Sn2 fatty acid is linked via an acyl bond, whilst the Sn1 fatty acid is attached by a vinyl ether bond [96]. In platelets, plasmalogens with an ethanolamine linked head-group are abundant and are reported to represent approximately 12% of total membrane phospholipids [97] [98]. The vinyl ether bond is particularly vulnerable to reactive oxygen species, thus implicating a protective role for plasmalogens in limiting oxidative damage to other lipid species and cells [98] [99]. It is hypothesised that oxygen free radicals generated from platelets or other blood cells may induce platelet aggregation and contribute to thrombus formation for example in atherosclerotic plaques [100] [101]. Whether the antioxidant properties of plasmalogens are of significance in this disease process remains to be elucidated.

1.4.2. Role of the phospholipid platelet membrane in coagulation

The phospholipid composition of the membrane plays an essential role in coagulation. In the resting state, the phospholipid membrane is maintained in an asymmetrical configuration. The outer leaflet is mainly composed of phosphatidylcholine (PC) and sphingomyelin, whereas phosphatidylethanolamine (PE) and phosphatidylserine (PS) are located in the inner surface of the membrane [102]. The distribution of mostly neutral phospholipids in the outer membrane and negatively charged lipids in the inner membrane creates an electrical gradient which is regulated by transmembrane lipid transporters. ATP-dependent translocase or flippase facilitates the transport of PS and PE from the outer to the inner membrane. A second ATP-dependent mechanism due to floppase activity, regulates the movement of predominantly PC to the outer membrane [103]. The export of lipids in the manner described is associated with the ABC transporter superfamily. Of those members characterised, ABCB4 is identified as having specificity for the outward translocation of PC [104] [105].

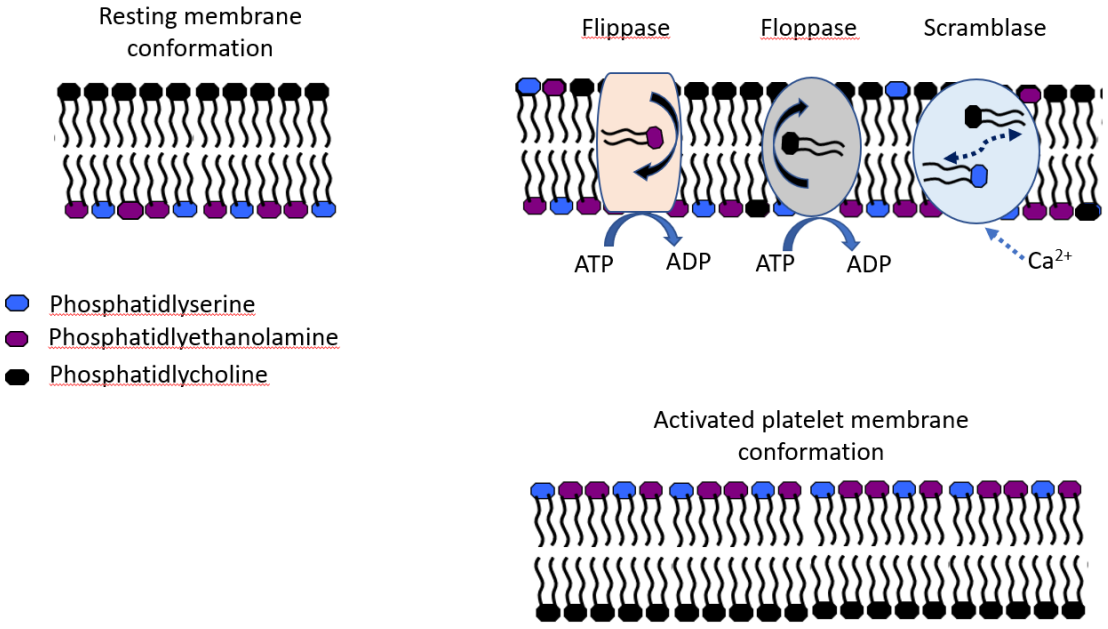


Figure 1.11 Regulation of phospholipid membrane asymmetry. The action of the three transporters involved in the movement of phospholipids between the inner and outer membrane is shown. Flippase and floppase both require ATP to move phospholipids in a unidirectional manner. Scramblase activity is bidirectional and is calcium dependent. The activated platelet membrane externalises PS and PE.

Upon platelet activation or apoptosis, membrane lipid asymmetry is disrupted due to the rapid passage of negatively charged PE and PS to the outer surface. This is induced by calcium-dependent scramblase activity which mediates the bidirectional movement of phospholipids. At the same time, the increased intracellular calcium renders both flippase and floppase inactive [102, 103]. The net effect of these processes is the surface exposure of PS and PE. At the surface, they are available to interact with the negatively - charged Gla domains of the Vitamin K dependent coagulation factors (II, VII, IX, X). It is currently thought that the binding of calcium ions to the Gla domain exposes a hydrophobic region within the omega loop which can then penetrate the anionic phospholipid membrane [106]. Each Gla domain has a single binding site specific for the PS head-group in addition to binding sites for interactions with the phosphates on any phospholipid apart from PC. Nuclear magnetic resonance (NMR) analysis has shown that the PL head-group must bend to enable its phosphate to associate with calcium bound within the Gla domain. Whilst PE can engage in phosphate-specific interactions and thus enhance the function of PS, it is hypothesised that PC may impede these interactions due to the bulky nature of the choline head-group [106] [107]. The phospholipid-binding properties of the non-vitamin K dependent co-factors (FVIII and FV) resides within the C domain. Specifically, binding sites have been identified in the C2 domain which contains a 'β2 barrel framework' with hydrophobic residues [108] although additional sites have been identified in the C1 domain [109] [110].

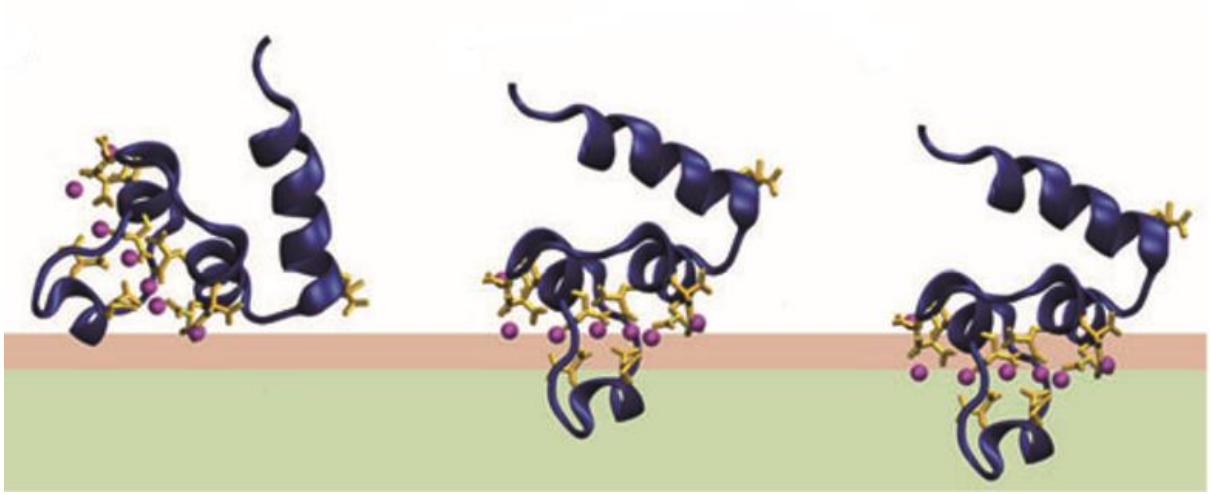


Figure 1.12a Gla domains binding to the phospholipid membrane. The omega loop of the Gla domain of prothrombin (blue) is shown interacting with the external leaflet of the membrane. The hydrophobic residues of the omega loop (yellow) are exposed and calcium (purple) is tightly bound within the Gla domain. Figure used with permission from Morrissey JH, Tajkhorshid E and Rienstra CM. Nanoscale studies of protein- membrane interactions in blood clotting. Journal of Thrombosis and Haemostasis, 2011. 9:163 [111].

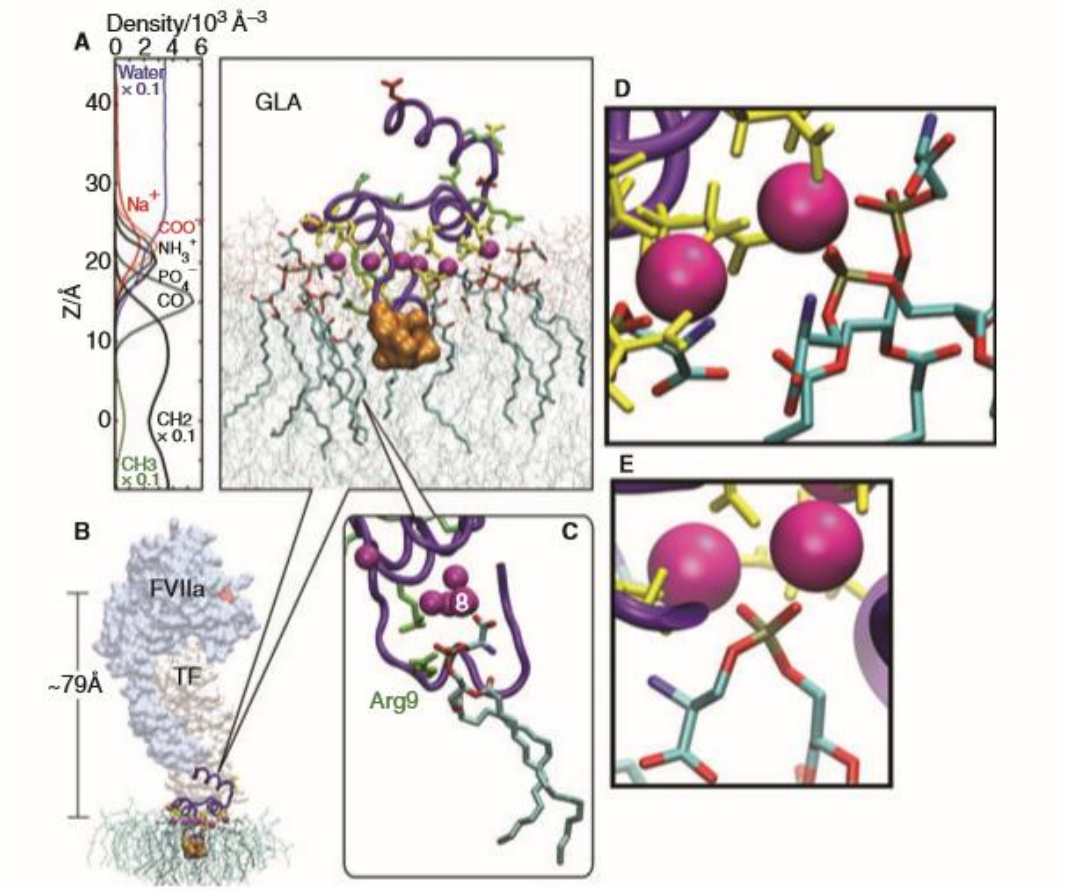


Figure 1.12b Membrane interactions of the Gla domain of FVIIa. (A) The residues of the Gla domain (yellow) of FVIIa is shown interacting with exposed PS on the membrane surface. **(B)** The TF-FVIIa complex is membrane associated **(C)** Binding of PS (green stick representation) to the Gla domain (dark purple) with its head-group in contact with calcium (pink/purple). **(D-E)** Interaction of calcium bound within the Gla domain and the phosphate groups (red) of PS. Figure used with permission from Morrissey JH, Tajkhorshid E and Rienstra CM. Nanoscale studies of protein- membrane interactions in blood clotting. Journal of Thrombosis and Haemostasis, 2011. 9:164 [111]

Activated platelets undergo blebbing and shedding of the plasma membrane forming microvesicles. This process is dependent upon an increase in intracellular calcium which also induces scramblase activity and is associated with degradation of cytoskeleton proteins mediated by calpain [112] [113]. Microvesicles are small lipid particles (100 nm- 1 µM) which have been demonstrated to express externalised PS/PE by Annexin V binding and thus possess procoagulant properties[114]. Their accumulation at the site of clot formation facilitates the assembly of coagulation complexes on their surface thus promoting the generation of thrombin [115]. The presence of active TF-bearing microvesicles derived from cells such as monocytes in response to lipopolysaccharide (LPS) is well established [116], however the role of platelets as a source of TF, and thus as a source of TF-positive microvesicles has been debated. Whilst some studies have reported the presence of platelets expressing TF in response to stimulation with an

agonist [117, 118], others have failed to demonstrate these findings [119, 120]. Currently, the most widely held concept involves the integration of TF into the platelet membrane from TF-bearing microvesicles derived from cells such as monocytes and endothelial cells[121]. Alternative mechanisms that have been proposed include the transfer of TF mRNA from the megakaryocyte to a subset of platelets during their production [122].

1.4.3. Arachidonic acid metabolism in platelets

Arachidonic acid metabolism is of importance in platelets as this lipid is a precursor for the generation of several lipid mediators. Following platelet activation, arachidonic acid from membrane PL is hydrolysed from the Sn2 position by phospholipase A2 (PLA2). Of the several isoforms, cytosolic group IV PLA2 (cPLA2) shows specificity for phospholipids containing arachidonic acid at the Sn2 position [123] and is regulated by calcium and kinases including mitogen-activated protein kinase (MAPK) [124]. Free arachidonic acid can be utilised in the cyclooxygenase (COX) pathway leading to the production of prostaglandin G2 (PGG2) and then prostaglandin H2 (PGH2). The COX enzyme family consists of two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in human platelets in contrast to COX-2, an inducible isoform which is activated in response to inflammatory stimuli [125]. PGG2 and PGH2 compounds are converted by thromboxane synthetase into thromboxane A2 which in addition to its function as a platelet activator also mediates smooth muscle contraction [126]. Other arachidonic acid derived oxylipins (PGE_2, PGD_2) which regulate the response to inflammation and hypersensitivity are also generated by platelets, but in far less amounts [127].

The second pathway of arachidonic acid metabolism in platelets involves lipoxygenases (LOX), a group of non-heme iron-containing enzymes [128]. LOX enzymes oxidize arachidonic acid forming hydroxyperoxyeicosotetranoeic acids (HPETEs) which are rapidly reduced by glutathione peroxidases to form hydroxyeicosotetranoeic acids (HETEs). The oxidation reaction catalysed by LOX involves hydrogen abstraction followed by radical migration and subsequently the stereospecific addition of oxygen at a particular carbon, dictated by the LOX isoform [129]. LOX isoforms are numbered in accordance with the carbon position at which arachidonic acid is oxygenated [130]. In human platelets, the formation of 12-HETE results from the enzymatic oxidation of arachidonic acid by 12-LOX [131-133]. 12-HETE can either be released by activated platelets or be esterified generating oxidised phospholipids (OxPLs) [134].

5-LOX in human leucocytes (neutrophils/monocytes) and its products are used for the downstream production of leukotrienes which have potent inflammatory actions [130]. Two different subtypes of 15-LOX have been described in humans, 15-LOX-1 (leukocyte-type) and 15-LOX-2 (epidermis-type)[131] [132]. Of these isoforms, 15-LOX-1 has the wider distribution of the two and is expressed in reticulocytes, eosinophils, bronchial airway epithelial cells and skin [135]. Peripheral blood monocytes, however express 15-LOX-1 when exposed to anti-inflammatory cytokines such as IL-4 and IL-13 [136] [129].

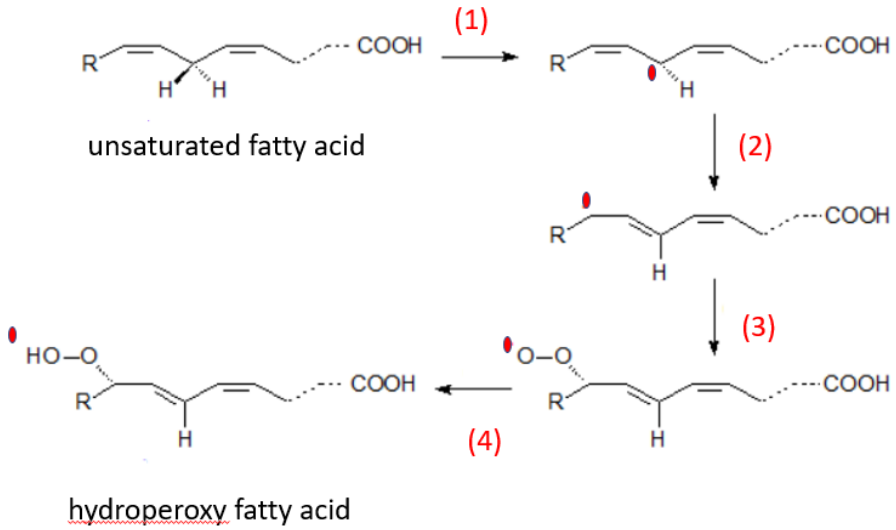


Figure 1.13a Mechanism of the lipoxygenase reaction. (1) - removal of hydrogen from the unsaturated fatty acid (hydrogen abstraction) (2) – radical rearrangement (3) addition of oxygen at the site of lipoxygenase specificity and (4) reduction of the peroxyl radical to form the hydroperoxyeicosotetranoeic acid (HPETE) within the enzyme active site. Figure used with permission from http://www.lipidhome.co.uk/lipids/fa-eic/eic-hete/index.htm

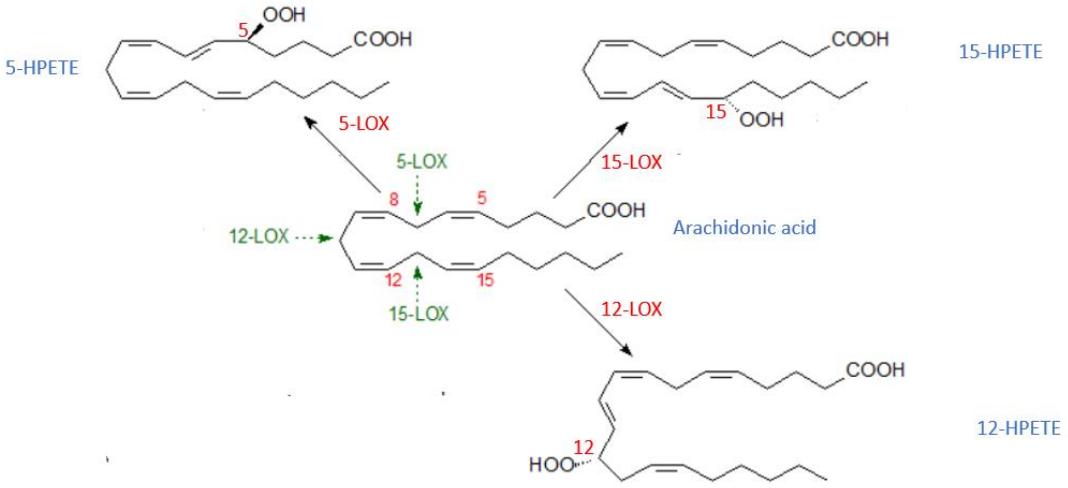


Figure 1.13b Examples of LOX specificity at different positions of arachidonic acid. The carbon number is denoted in red. The initial position of the removal of hydrogen (abstraction) is arrowed in green and differs to the carbon position at which arachidonic acid is oxygenated by LOX. Figure used with permission from http://www.lipidhome.co.uk/lipids/fa-eic/eic-hete/index.htm

1.4.4 Enzymatic and non- enzymatic formation of oxidised phospholipids

The oxidation of a polyunsaturated fatty acid (PUFA) such as arachidonic acid can occur by enzymatic or non-enzymatic mechanisms either when they are free or attached to PLs [137]. The non-enzymatic oxidation of PL can be initiated by free radicals or reactive oxygen species generated during inflammation for example. The initial reactions involved in non-enzymatic PL oxidation produce peroxyl radicals which can react with the methylene group in other PUFA molecules to form hydroperoxides and other carbon centred radicals. Further secondary peroxidation reactions can then occur in an enzyme-independent manner leading to the generation of a wide variety of oxPL products [137]. The biological effects of non-enzymatically formed oxPL have been associated with the development of human atherosclerotic plaques due to their influence on vascular endothelial cells and macrophages [138-140].

Despite being derived from the same initial set of chemical reactions (Figure 1.13a), a clear distinction is made with enzymatically formed OxPLs regulated by COX and LOX enzymes. The stereospecific activity governed by a particular LOX isoform results in the generation of a less diverse number of molecular species [141]. Over the last two decades, a number of HETE-PL species generated by LOX enzymes have been identified following the cellular activation of human monocytes, neutrophils and platelets [134, 142, 143]. Although their role has not fully elucidated in health or disease, there is increasing evidence that the families of eoxPL generated in this manner are important in mediating the inflammatory response and promoting coagulation [141].

It is well established that large amounts of 12-HETE are generated and released from platelets upon agonist activation [134, 142]. For example, collagen and collagen related peptide (CRP) activate 12-LOX via GPVI signalling inducing the production of 12-HETE [144]. More recently it has been observed that other platelet agonists including thrombin, convulxin and ionophore rapidly generate free 12-HETE [134]. Stable isotope labelling demonstrated that of the total 12-HETE synthesised, approximately 30% is esterified to PLs and remains cell-associated whilst the remaining free 12-HETE is secreted from the platelet [134]. The mechanisms involved in agonist-activation of 12-LOX include intracellular signalling through PI3 kinase activation and calcium mobilisation [144]. The six molecular species of 12-HETE-PLs produced by activated platelets include four molecular species of 12-HETE-PE (PE(18:0a_12-HETE), PE(18:0p_12-HETE), PE(18:1p_12-HETE), PE(16:0p_12-HETE)) and two species of 12-HETE-PC (PC(18:0a_12-HETE), PC(16:0a_12-HETE)). Their identification has been observed within minutes and is sustained for up to 3 hours [134].

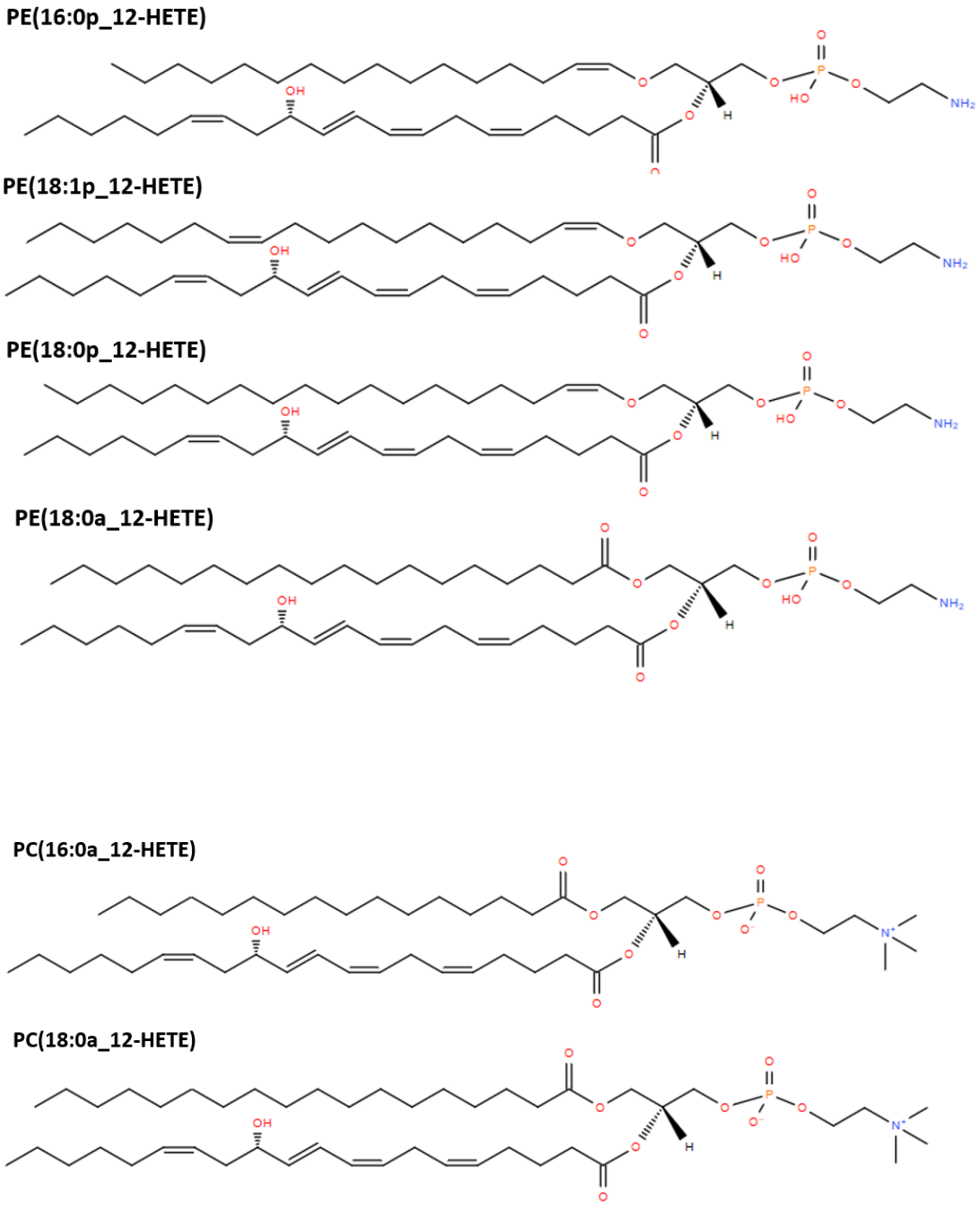


Figure 1.14 Structure of 12-HETE-PEs and 12-HETE-PCs generated by activated human platelets. Images constructed using LIPIDMAPS. www.lipidmaps.org/resources/tools/index.php

1.4.5. eoxPL and coagulation

There is an increasing body of evidence to support the idea that 12-HETE-PLs have thrombotic effects [145]. In thrombin generation experiments activated by TF-containing liposomes in platelet poor plasma, the rate and amount of thrombin produced was increased by incremental amounts of 12-HETE-PE (ranging from 1-10% of total lipid composition) when compared to unoxidized PE (30% of total lipid composition). Interestingly, similar findings were observed when 12-HETE-PC (ranging from 1-10%) was substituted for the native form (65% of total lipid composition) [146]. This is in contrast to native unoxidized PC which does not support coagulation [102, 107]. In a reconstituted coagulation system of physiological concentrations of coagulation factors (II, V, VII, IX, X), 12-HETE PE and 12-HETE-PC both enhanced the formation of the tenase and prothrombinase complexes which were triggered in TF-dependent manner [146]. As native PC is generally found on the outside of membranes, it is proposed that 12-HETE-PCs are also located on the external leaflet where they can exert procoagulant activity. The incorporation of the HETE hydroxyl-group (OH) into arachidonate facilitates a change in configuration of the PL to a bent form which enables its interaction with calcium and the phosphate groups of other lipids or the carboxylate on the PS head-group [134, 146]. It has been shown by molecular modelling that due to the increased polarity introduced by the -OH group, 12-HETE-PCs can associate with the outer part of the membrane where they can contribute to the electronegative charge in addition to generating space between head-groups for calcium to bind [146].

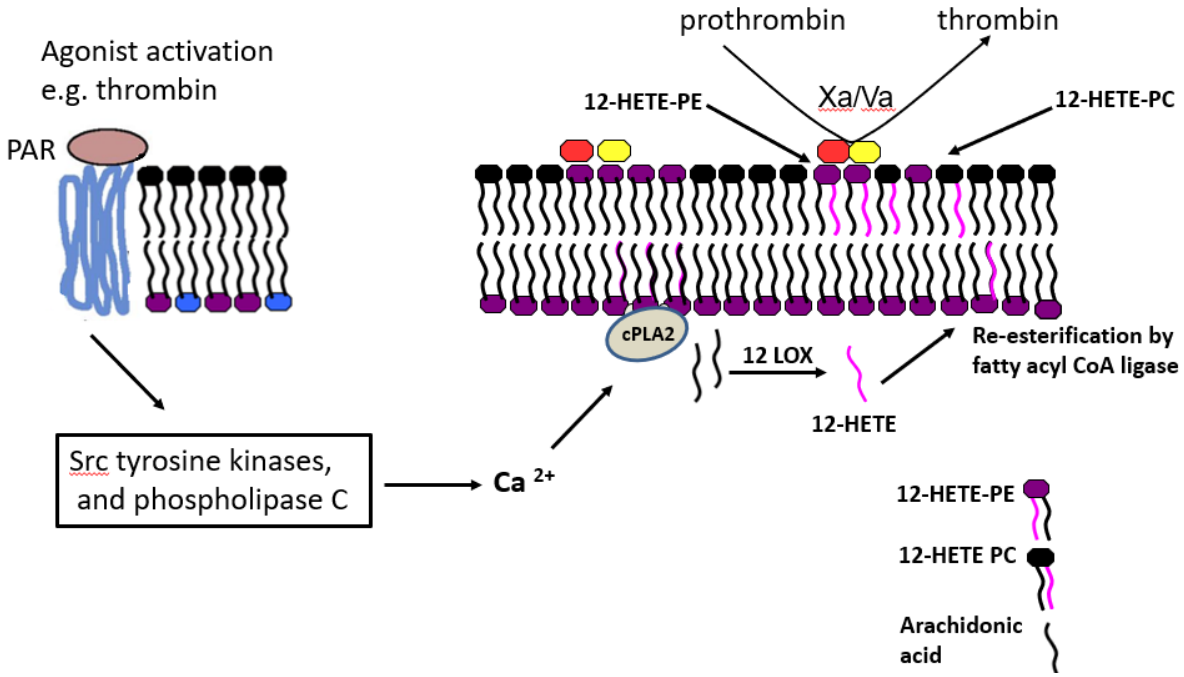


Figure 1.15a Mechanism of 12-HETE- PE and 12-HETE-PC formation in platelets. Following thrombin activation of the PAR receptor, synthesis of 12-HETE occurs via signalling pathways involving Src tyrosine kinase, phospholipase C and calcium. The formation of 12-HETE-PL involves hydrolysis of arachidonate by cPLA2, oxidation by 12-LOX and then re-esterification by fatty acyl- CoA ligase. Some 12-HETE-PE moves to the outside membrane where it can promote coagulation. 12-HETE-PC is located on the external membrane leaflet. Figure adapted from O'Donnell et al[147] [148].

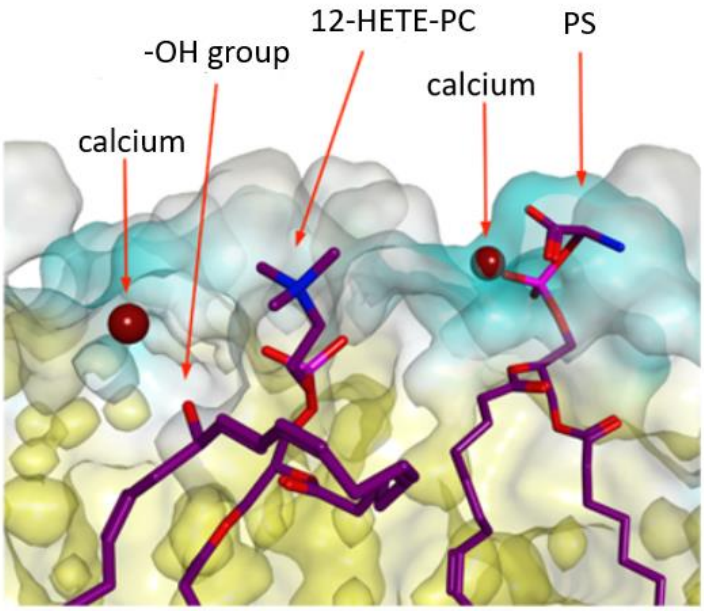


Figure 1.15b Molecular dynamics simulation image showing the association of the -OH group of 12-HETE-PC with the outer polar membrane. The bent-up configuration of 12-HETE-PC is shown where the -OH group is close to the polar membrane where it can interact with the phosphate groups of other lipids and the nearby phosphatidylserine (PS) head-group. From Lauder SN et al. Networks of enzymatically oxidised membrane lipids support calcium-dependent coagulation factor binding to maintain haemostasis. Science Signalling, 2017, 10(507). Reprinted with permission from AAAS [146].

In addition to the above findings, in vivo murine experiments using a tail bleeding model demonstrated that mice deficient in 12/15 LOX or 12-LOX had increased bleeding times compared to their wild-type counterparts. Importantly, injection of 12-HETE-PE liposomes significantly shortened the bleeding time compared to unoxidized PE. Subsequently, platelets from patients with antiphospholipid syndrome (n=12) generated increased levels of 12-HETE-PE compared to healthy controls (n=18) both basally and after thrombin stimulation ($P < 0.05$) [146]. Finally, it was demonstrated that measured levels of 12-HETE -PE and 12-HETE-PC were lower in the platelets of patients post cardio-pulmonary bypass surgery compared to pre-operatively [149]. Such patients are recognised to be susceptible to bleeding in this clinical scenario for many reasons including contact activation, acquired platelet defect or from anticoagulation, and it was hypothesised that decreased levels of oxPL may be contributory.

1.4.6. Native aminophospholipids (APL)

The externalisation of PE and PS that occurs during agonist activation, apoptosis or ageing has traditionally been measured using flow cytometry techniques using annexin V or lactadherin [150-152]. Whilst these techniques have been useful in establishing the presence of APL, they are unable to distinguish different species or their specific quantities. Following the development of a new lipodomics method using a cell impermeable reagent, it was demonstrated that human platelets undergoing activation, ageing or energy depletion could externalise 5 species of PE and 3 species of PS [95, 153]. The importance of calcium dependent scramblase activity in PE/PS exposure has previously been shown in the rare bleeding disorder, Scott syndrome where this mechanism is impaired due to a causative TMEM16F mutation [154]. The platelets of such patients externalise significantly reduced quantities of PE and PS, which is recognised to account for the bleeding phenotype [134, 155].

It has been suggested, however that whilst TMEM16F is required for scramblase activity triggered by platelet agonists such as collagen and thrombin, other independent mechanisms may account for PS exposure due to apoptosis [155]. It has been shown that platelets contain proteins including members of the BCL-2 family which regulate apoptosis and caspases (caspase 3 and 9), which mediate programmed cell death [156] [157, 158]. Subsequently, inhibition of the anti-apoptotic protein Bcl-XL in platelets, was reported to result in mitochondrial damage, activation of caspase and exposure of PS [159-161]. Whether there is an association between the apoptotic pathway and agonist-activated mechanisms for PS exposure is currently undetermined [158, 159]. Apart from Scott syndrome, there have been no investigations into potential variations in externalised PE and PS in bleeding or thrombotic disorders.

Recently, it was reported that increased PE exposure was observed on the surface of platelets and eosinophils of *Alox12*- or *Alox15*- deficient mice. In a murine model of Angiotensin-II driven aortic abdominal aneurysm (AAA), both 12- and 12/15- LOX deficient mice demonstrated evidence of a consumptive coagulopathy as confirmed by increased prothrombin time and higher thrombin-antithrombin complexes (TAT's) compared to wild type. Whilst these mice generate less procoagulant oxPL compared to wild - type, increased basal quantities of externalised PE were measured in platelets and in eosinophils following activation with ADP. This was hypothesised to be a compensatory mechanism for the underlying coagulation defect [162].

1.4.7. Phospholipid remodelling

The main remodelling pathway of phospholipids is known as the Lands cycle which was initially described in 1958 [163]. This process is initiated when the fatty acid chain at the Sn2 position is hydrolysed by phospholipase A2 generating a PUFA such as arachidonic acid, and a lyso-phospholipid for example lyso-PC or lyso-PE. An important group of enzymes, long-chain acyl-CoA synthetases (ACLS) act on the long-chain fatty acid substrate generating a product (acyl-CoA) that is used by other membrane bound enzymes known as lysophospholipid acyltransferases (LPLATs) [164]. LPLATs esterify the acyl-CoA product onto a lyso-PL acceptor and can therefore re-introduce a different fatty acid at the site of cleavage thus influencing the phospholipid composition of the membrane [165].

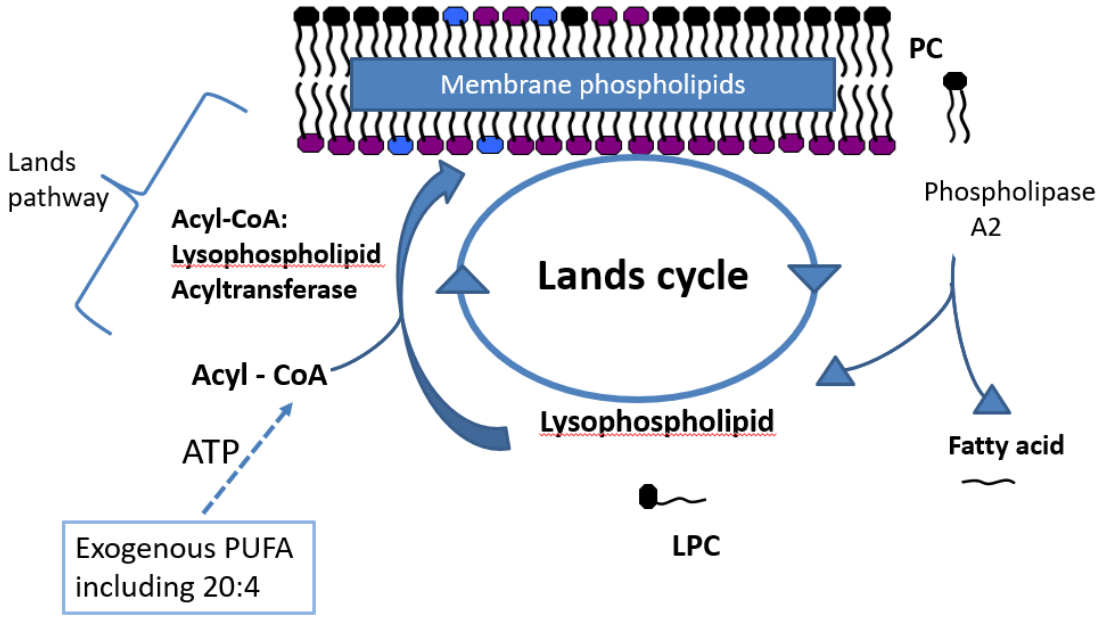


Figure 1.16. Lands cycle Membrane phospholipids are remodelled through the actions of phospholipase A2 (PLA2) and lysophospholipid acyltransferase (LPLAT). As an example, PLA2 removes a fatty acid (arachidonic acid; 20:4) from the Sn2 position of phosphatidylcholine (PC) to produce lysophosphatidlycholine (LPC). The fatty acid can be replaced by either the same or a different fatty acyl group depending on the specificity of lysoPC acyltransferase (LPCAT). LPCAT uses fatty acyl-CoA as a donor. Adapted from Sugiura et al [166].

Lysophosphospholipid acyl-transferase (LPLAT) enzymes are mainly involved in the acylation of lyso-PC, lyso-PE and lyso-PS [167]. By virtue of their name, these enzymes demonstrate a degree of specificity with regards to their lysophospholipid substrate and acyl-CoA donor of choice. For example, LPCAT demonstrates substrate specificity for lyso-PC (Figure 1.16) in a similar manner to lyso-PE acyltransferase (LPEAT) for lyso-PE, however the enzymatic activity of each individual LPLAT is not restricted to a specific substrate [163]. The LPLATs were recently further classified following the identification of two protein families, the acylglycerophosphate acyltransferases (AGPAT) and the membrane bound O-acyl transferases *(*MBOAT) [168]. The majority of LPCATs belong to the MBOAT family, which were identified from 11 genes of the human genome [93]. MBOAT activity occurs mainly within the endoplasmic reticulum or mitochondria, although the activity of the different isoforms is distributed throughout a variety of tissues.

It is hypothesised that the availability of arachidonic acid for the COX and LOX pathways is regulated by the deacylation and reacylation cycle of phospholipid remodelling. This concept was demonstrated in human neutrophils where inhibition of MBOAT activity led to over a 50-fold increase in leukotriene production [169]. It is well-established that two human MBOATs (MBOAT 5 and MBOAT 7) preferentially utilise arachidonyl-CoA as an acyl donor [93, 170]. Previous studies have also shown that MBOAT5 (or LPCAT 3) has the most specificity for lyso-PC as an acyl receptor followed by lyso-PS and then lyso-PE. It has therefore been suggested that MBOAT5 has an important role in incorporating arachidonyl acid into lyso-PC, -PS and -PE [169].

The second identified human MBOAT using arachidonyl-CoA, MBOAT 7 (or lyso-PI acyltransferase 1 (LPIAT1)) has been reported to have a far more limited substrate specificity for lyso-PI [170]. Whilst these observations have been reported in human neutrophils, it remains to be determined whether they are applicable to human platelets [165]. Furthermore, the role of these enzymatic pathways in the generation of eoxPL in platelets and neutrophils is currently unknown.

1.5 Bleeding disorders

Inherited bleeding disorders can be broadly sub-divided according to defects in primary haemostasis, secondary haemostasis or fibrinolysis. Often, the nature and presentation of bleeding may suggest an underlying cause which can be confirmed with specific laboratory investigations. Mucocutaneous bleeding, for example is well recognised as a characteristic feature of primary haemostatic disorders prompting laboratory investigations for Von Willebrand Disease or platelet function tests [171].

The multifunctional role of platelets provides many opportunities for an abnormality to manifest. These include (i) disorders of platelet adhesion e.g. Bernard Soulier Syndrome characterised by a defect or deficiency of GP Ib-V-IX complex (ii) aggregation defects e.g. Glanzmann's thrombasthenia due to abnormal or reduced amounts of the GP IIb/IIIa receptor and (iii) secretion disorders including signal processing defects and storage pool disorders. In addition, platelet receptors for the soluble agonists may be defective or specific abnormalities may occur in arachidonic acid metabolism, for example due to cyclo-oxygenase or thromboxane synthase deficiency. Several genetic defects associated with the platelet cytoskeleton such as the MYH-9 and Wiskott-Aldrich syndrome disorders have been characterised [172-174].

Clinical suspicion of a secondary haemostatic disorder is confirmed by a reduction in one or more coagulation factors. Of the inherited bleeding disorders, the most prevalent are Haemophilia A and B caused by deficiency in Factor VIII and IX respectively. Patients with severe haemophilia display a spectrum of haemorrhagic manifestations including spontaneous intra-articular bleeds. Generally, the severity of bleeding symptoms correlates with a decrease in the coagulation factor level, although it is recognised that this is not entirely applicable to Factor XI deficiency [175]. Disorders of fibrinolysis are typically characterised by delayed or late-onset bleeding, but diagnosis is compromised by the lack of high-quality diagnostic tests. They are unusual in that both haemorrhage and thrombosis may occur for example in deficiencies of the fibrinolytic inhibitors such as PAI-1 [176].

The standard tests of primary and secondary haemostasis are often normal in bleeding disorders associated with vascular abnormalities such as hereditary haemorrhagic telangiectasia and collagen disorders for example Ehlers Danlos syndrome. Clinical manifestations of these disorders are usually identifiable however and can be confirmed with genetic testing [177, 178]. Defects of platelet procoagulant activity may also have no demonstrable abnormality when investigated with standard platelet function tests or coagulation factor assays. Historically, the prothrombin consumption index (PCI), was used as a measure of the availability of platelet membrane phospholipid in coagulation. This laboratory test compares the ability of serum and plasma to form a clot by measuring the conversion of prothrombin to thrombin [179]. Scott syndrome is a well described although exceptionally rare inherited disorder of platelet procoagulant function, characterised by mild to severe bleeding following a haemostatic challenge. In this condition there is a failure of platelets to externalise PS/PE and impaired shedding of microvesicles due to defective scramblase activity with causative mutations found in the gene encoding TMEM16F [153, 154]. Whether other subtle differences in platelet

phospholipid composition or externalisation may contribute to bleeding disorders of unknown cause has not previously been investigated.

1.6. Unclassified bleeding disorders

In a subset of patients with a significant bleeding history, no reproducible abnormality is identified despite extensive laboratory testing. Such cases are defined as unclassified bleeding disorder (UBD). Although the true incidence is unknown, 588 patients in the UK were formally registered with UBD in the 2017 and 2018 annual returns and UBD was therefore reported to account for 5.3% of rare bleeding disorders [180]. Other reports however suggest that the incidence of UBD is significantly higher with such cases representing up to 60% of mild inherited bleeding disorders [181]. This discrepancy is likely to result from a lack of standardised criteria for diagnosis and the varying number of global assays available to the testing coagulation laboratory. Additionally, it is recognised that the diagnosis of UBD is not clear- cut largely due to lack of sensitivity of routine assays to detect abnormalities of fibrinolysis for example, and difficulties in the interpretation of mildly abnormal screening tests or platelet aggregation in the context of a subtle rather than a severe defect [182, 183]. Furthermore, other potential factors such as vessel wall fragility may be contributory. Although not a new or uncommon clinical finding, reports regarding the diagnosis, management and the underlying pathophysiology of UBD are extremely scarce. The ongoing NIHR Bioresource Rare Diseases BRIDGE BPD study was the first to investigate this cohort of patients with the aim of identifying the genetic basis of unresolved bleeding and platelet diseases by exome-sequencing. It is reported that testing with a gene panel of 87 genes is informative in approximately 3.2% of UBD cases, although not all of patients would have been diagnosed using the same criteria [7]. Apart from BRIDGE-BPD, there have been only limited attempts to investigate the cause of bleeding in this cohort of patients to date.

The first study to report on the investigation of UBD and the outcomes of management was from our Cardiff Haemophilia Centre in 2016 [184]. Currently no standardised diagnostic criteria exists, however the initial characterisation of UBD was based upon a positive bleeding history as defined by the International Society of Haemostasis and Thrombosis (ISTH) bleeding assessment tool and a minimum set of standard coagulation tests: -

i) ISTH-BAT score ≥4 men, ≥6 women.
ii) Normal platelet count (150 – 400 x 10^{9}/l)
iii) Normal coagulation profile (prothrombin time (PT), activated partial thromboplastin time (APTT) and Clauss fibrinogen
iv) Platelet function studies (PFA-100, platelet aggregation studies and nucleotides (total platelet content)
v) Von Willebrand (VWD) screen (minimum of von Willebrand factor RiCof, VWF antigen)
vi) Factor assays (VIII, XIII, XI) and IX.

In the above report of 33 UBD patients (28 females and 5 males), the mean age of diagnosis was 45.2 years (16-83 years). A family history of bleeding was confirmed in 48% of cases implying a heritable cause. The bleeding scores were significant in that 3 patients had an ISTH-BAT score between 16-20 and the remaining patients had a bleeding score between 5-10 (15 patients) and 11-15 (15 patients). The mean bleeding score was 9.9 (SD 3.3). The most common haemorrhagic symptoms were menorrhagia (89%), bleeding post-surgery (88%) and bleeding following a dental extraction (85%). Although the mechanism of action is unclear in this group of patients,

desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP) and tranexamic acid (lysine analogue) were effective haemostatic agents. In 70 out of 78 haemostatic challenges, no bleeding was observed using this treatment regimen [184].

In a recent larger cohort of 124 patients with UBD from the Cambridge Haemophilia Centre, 91% of patients were female demonstrating a predominance consistent with the report above. Bleeding scores however were lower with the mean score reported as 8.8 (SD 3.8) and then 6.4 following the deduction of menorrhagia and post-partum haemorrhage using an alternative bleeding assessment tool (condensed MCMDM1VWD Bleeding Questionnaire). This suggests that the study population may have had a comparatively milder bleeding phenotype. Of note, the investigators were unable to characterise patients with UBDs using viscoelastic haemostatic assays or using thrombin generation assays on platelet poor plasma. Empirically, tranexamic acid and DDAVP were reported to be effective at preventing bleeding in 69 procedures and 13 deliveries [185].

DDAVP, a synthetic form of the human hormone L-arginine vasopressin has traditionally been used for its anti-diuretic effect via its action on V2 receptors in the renal collecting duct [186]. The release of VWF from Weibel-Palade bodies is also mediated through V2 receptors on endothelial cells and cyclic adenosine monophosphate (AMP) dependent signalling [187]. The mechanism for the secondary elevation (approximately 3-fold) of FVIII is not well-defined, however has widened the therapeutic use of DDAVP as a haemostatic agent in mild Haemophilia A as well as certain sub-types of VWD. In addition, DDAVP is used in congenital and acquired platelet defects, although its precise mode of action is unclear since the VWF and FVIII levels are normal in these patients. It has been proposed that the increased levels of VWF following DDAVP administration may enhance platelet adhesion and that the subsequent release of ultra-large VWF multimers enables this process to occur in conditions of high shear with further procoagulant effects from supra-normal levels of FVIII [188]. More latterly, the haemostatic effects of DDAVP in platelet function disorders has been attributed to the increased generation of COAT platelets demonstrated by enhanced surface expression of PS detected with Annexin V. Furthermore, platelet-dependent thrombin generation was also observed to increase with enhanced intracellular sodium and calcium mobilisation proposed as a part of the underlying mechanism [51]. It is unknown whether impaired platelet procoagulant activity underpins the pathophysiology of UBD or explains the efficacy of DDAVP as a haemostatic agent in UBD.

1.7 Thrombosis

The clinical manifestations of venous thromboembolism (VTE) include pulmonary embolism (PE) and deep vein thrombosis (DVT). Of these cases, two-thirds are due to DVT most often involving the lower extremities, and PE accounts for the remaining third. VTE is a relatively common disease with an annual incidence of 1 in 1000 adults, although the risk increases with advancing age with estimates rising up to 7 per 1000 in those aged 70 years or above [189, 190]. It is reported to be the third leading cause of cardiovascular morbidity after ischaemic heart disease and stroke [191] and accounts for approximately 500,000 deaths per year in Europe [192].

Several inherited or acquired prothrombotic risk factors often contribute to a venous thrombotic event. More than one risk factor is typically identified in over two thirds of patients [193]. Acquired risk factors are by far the most common and include physical factors such as inactivity due to recent major surgery, trauma or hospitalisation. Diseases such as malignancy, inflammatory conditions and acquired thrombophilic defects for example antiphospholipid syndrome are also associated with VTE as well as hormonal effects including pregnancy and oral

contraceptives. Hereditary thrombophilic defects include factor V Leiden mutation, prothrombin G20210A mutation, antithrombin, Protein C and Protein S deficiency [194]. In approximately 30% of patients however, a causal risk factor cannot be identified and these unexplained cases are classified as idiopathic or unprovoked VTE [195].

Virchov's triad proposes that VTE occurs as a result of blood stasis, hypercoagulability and vascular endothelial damage [196]. The mechanisms for the formation of deep vein thrombi are recognised to differ from that of arterial thrombi. Post-mortem studies report the characteristic red cell-fibrin rich thrombus that forms on an undisrupted endothelium in sinuses near venous valves with finely interspersed white lines of platelet-fibrin units commonly referred to as the 'lines of Zahn' [197]. It is hypothesised that the hypoxic micro-environment promoted by blood stasis in valve sinuses may down-regulate anti-thrombotic proteins expressed on venous valves, namely thrombomodulin and endothelial protein C receptor [198]. Additionally, hypoxia is recognised to enhance the expression of P-selectin, potentially on the endothelium, which could trigger the recruitment of leucocytes, platelets and other inflammatory cells or microvesicles bearing tissue factor. It is widely accepted that both tissue factor and P-selectin are essential for thrombus formation, although their exact source of origin is debatable [198, 199] [200]

Although platelets have been implicated in the pathophysiology of arterial rather than venous thrombosis, there is growing interest in the potential role of platelets in VTE disease. A recent review highlighted the association of VTE with genetic platelet polymorphisms [195]. Of these, polymorphisms in the GPIIbIIIa receptor are reported to be the most prevalent occurring in 35% of patients with PE (n=51) in one study compared with 14.3% of the control population (n=91)[200]. A well-known somatic acquired mutation in the Janus kinase 2 (JAK2) tyrosine kinase gene caused by a single amino acid mutation (Val617Phe) is found in up to 60% of patients with essential thrombocythaemia [201]. This myeloproliferative neoplasm which is characterised by increased clonal production of platelets is associated with a thrombotic risk greater than 20% [202]. Interestingly, higher levels of platelet P-selectin before and after agonist stimulation have been reported in ET patients compared to healthy controls including platelet-neutrophil and platelet-monocyte complexes [203]. More recently, higher levels of PS-exposing blood cells (platelets, erythrocytes, leucocytes) were demonstrated in JAK2 positive ET patients, thus implicating an alternative mechanism for thrombosis [204]. Whilst tumour cells and monocytes shed membrane particles rich in tissue factor, the procoagulant function of platelet derived microvesicles for example is well-established to be due to exposed PS. Elevated numbers of microvesicles have been associated with VTE, particularly in cancer patients compared with healthy controls [205]. It is currently unknown whether increased quantities of platelet membrane phospholipid may contribute to the occurrence of idiopathic or unexplained venous events.

1.8. Thrombin generation (Calibrated automated tomography)

The generation of thrombin is critical to clot formation and may therefore be of use in predicting an individual's bleeding or thrombotic risk. Standard coagulation tests such as the APTT and PT do not provide an accurate estimate of thrombin generation occurring in vivo since the end-point of clot formation is determined when less than 5% of the total thrombin has been produced [206]. Original attempts at measuring thrombin generation began in the 1950's and involved triggering coagulation in whole blood or plasma with sub-sampling over a time course into tubes containing fibrinogen solution. The clotting times of the fibrinogen solution were used to estimate the thrombin activity derived from calibration against a thrombin standard. This method however was considered both time-consuming and inaccurate [207].

Subsequent developments involved replacement of the fibrinogen solution with a chromogenic substrate. Measurements of colour change however, were influenced by the free thrombin available in addition to thrombin bound to α2-macroglobulin since both can cleave the chromogenic substrate. In contrast, the inhibition of thrombin by antithrombin involves binding to the active site of thrombin which renders the complex inactive [208]. An algorithm was therefore required to remove the contribution from thrombin bound to α2-macroglobulin. In this assay, the 'defibrination' of plasma was necessary to remove the limiting effect of fibrin on thrombin-α2-macroglobulin and to prevent turbidity [209]. This process however was recognised to significantly influence thrombin activity and the decay process thereby increasing experimental error [210].

A further development was the use of a slowly cleaved fluorogenic substrate, which removed the need for the defibrination step since the fluorescent product was not influenced by turbidity. Furthermore, this assay could be performed using platelet rich plasma, thus increasing its clinical utility [211]. The thrombin concentration could not be directly correlated with the fluorescent signal intensity due to substrate consumption and light absorption from other product molecules ('inner-filter effect') [209].

The calibrated automated thrombography (CAT) method devised by Hemker et al in 2003, circumvents the above by continuous monitoring of the separation of the fluorogenic substrate (Z-Gly-Gly-Arg-AMC) in parallel with a reference calibrator substance containing a known concentration of thrombin-like enzyme bound to α-2 macroglobulin in a parallel plasma sample [210]. Multiple experiments can be performed simultaneously using platelet rich plasma (PRP) or platelet poor plasma (PPP) with a trigger (tissue factor, phospholipids) followed by the addition of the fluorogenic substrate and calcium chloride. The thrombin generated cleaves the fluorescent substrate releasing a fluorophore. Measurements based on the velocity of the fluorescence increase (dF/dt) are taken from both test and calibrator wells and converted into thrombin concentrations (nM) by the Thrombinoscope software using a reference curve prepared by measuring the conversion rate of the fluorescent substrate with the calibrator substance. The CAT method utilises an algorithm which corrects for the activity of the α-2 macroglobulin-thrombin complex [210, 212].

The typical parameters measured from the thrombin generation curve include:-

(i) Lag time; the time period measured from the addition of a trigger to the initiation of thrombin generation
(ii) Peak thrombin; the peak height of the curve
(iii) Velocity index (VI); the peak height/(time to peak-lag time)
(iv) Endogenous thrombin potential (ETP); the area under the thrombin generation curve [206]

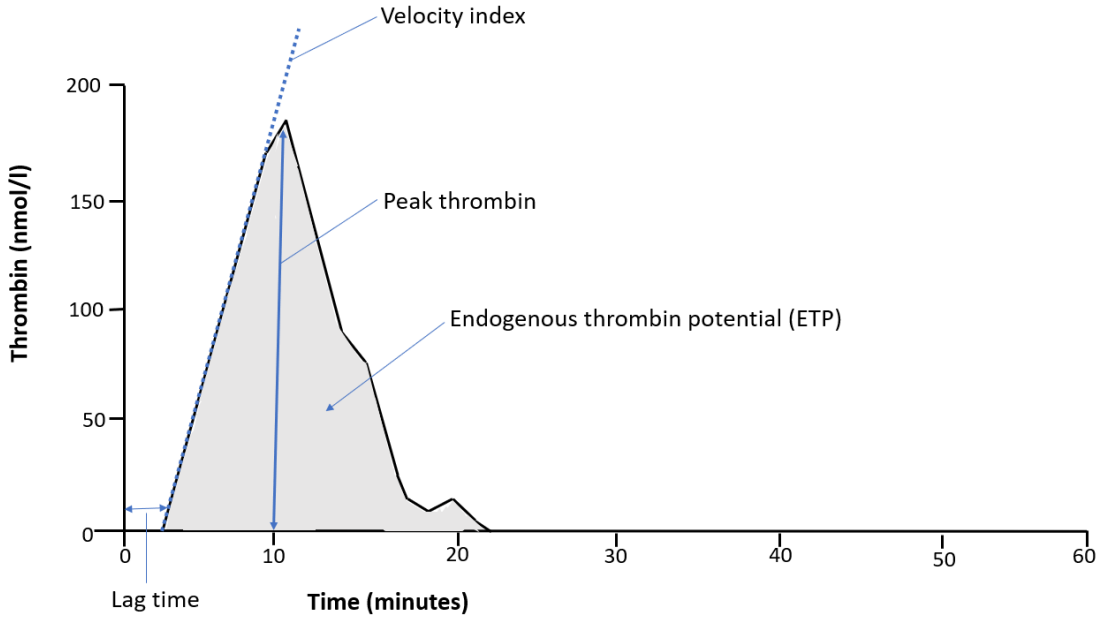


Figure 1.17 Schematic of a thrombin generation curve using calibrated automated tomography (CAT). The parameters measured include the lag time, the velocity index, the peak thrombin and the endogenous thrombin potential. Adapted from Montgomery et al [213]

Thrombin generation is dependent upon the available phospholipid, TF concentration and the source of plasma used. Currently, no recommendations exist to standardise the phospholipid or TF concentrations used in the thrombin generation assay leading to a degree of heterogeneity associated with its clinical use. At low concentrations of phospholipid (0-2 µM), a linear correlation of thrombin generation with phospholipid concentration is observed which is reported to plateau with concentrations of ≥ 3 µM. Standard studies performing thrombin generation assays with PPP have typically used a saturating concentration of 4 µM of phospholipid which makes the assay insensitive to phospholipid. Assays using PRP have generally omitted or used minimal concentrations of phospholipid which is supplied by the activated platelets [210, 214].

Most thrombin generation studies use concentrations of TF in the range of 3-6 pM (corresponding to 1/2000-1/1000 final dilution of recombiplastin) which are generally considered to be 'physiologically relevant' [207, 214]. It is observed that high concentrations of TF favours activation of the extrinsic pathway (Factor VII, Factor X) and overwhelms the effect of TFPI whereas lower quantities render the reaction more sensitive to deficiencies of the intrinsic pathway (Factor VIII, IX, XI) because TFPI inactivates the extrinsic pathway [215, 216]. For example, thrombin generation assays assessing the efficacy of replacement therapy in patients with haemophilia A and B, have used concentrations of TF ≤ 1 pM [217, 218]. Similarly, in Factor XI deficient patients the bleeding risk has been shown to correlate with thrombin generation using a trigger of only 0.5 pM TF [219] [220, 221].

Although most studies report the final concentration of TF in the thrombin generation assay, this information does not necessarily give information on TF function which is also dependent on the phospholipid membrane in which the TF is held. Therefore, to control the characteristics of the assay the TF concentration and the phospholipid environment need to be known and adjusted.

The requirement for the addition of corn trypsin inhibitor (CTI) at the point of blood collection has previously been debated. CTI suppresses activation of the contact pathway of coagulation by inhibiting Factor XIIa [222-224]. It has therefore been advocated that the addition of CTI is important when attempting to study the sole effect of the intrinsic tenase complex. It is usually recommended that CTI should be used in thrombin generation assays using triggers with low TF concentrations (≤ 1 pM) [220] [223].

1.9. Mass spectrometry analysis of platelet phospholipids

Both native and oxidised phospholipids described in this thesis were identified and quantified using mass spectrometry methods. For over five decades, MS has been an important tool in the field of lipidomics. Recent developments in the technique have enabled in-depth structural analysis of specific lipid species with respect to fatty acid side chain, back-bone and head-group [225].

Liquid chromatography (LC) is used to separate lipids prior to MS analysis based on their lipophilicity. Under high pressure, the lipid sample is injected into a mobile phase (water/polar solvent) which passes through an LC column containing the stationary phase, at a specific flow rate. Depending on the chemical interaction with the mobile and stationary phases, the lipids will elute from the column with varying retention times. The LC/MS/MS used in this thesis was the hybrid quadropole tandem mass spectrometer (ABI 4000 Q trap), thus enabling targeted and quantitative lipid analysis.

The fundamental principle of MS relies on the measurement of the mass-to-charge ratio *(m/z)* of a charged molecule. The advent of electrospray ionisation (ESI) in 1988 - widely referred to as 'soft ionisation' – has enabled the formation of intact gas-phase ions from liquid-phase molecules with minimal disintegration. This technique involves introducing the liquid analyte into the electrospray chamber through a hypodermic needle maintained at a high voltage. The small, charged droplets produced undergo a desolvation process and pass through a high vacuum, during which the neutral solvent-molecules are removed [226]. Depending on the ionisation mode selected, the positively $[M+H]^+$ or negatively $[M-H]^-$ charged ions pass through a linear series of three quadropoles. Each quadropole consists of four equidistant parallel rods through which an oscillating electric field is applied. The first quadropole (Q1), acts as a mass filter which is pre-set to separate ions based on the *m/z* of interest. The precursor or parent ions are then transmitted to a middle quadropole (Q2) where a neutral gas such as nitrogen, helium or argon is used to induce multiple collisions (collision-induced decompensation) resulting in fragmentation. Fragmented or daughter ions pass to the second mass filter (Q3) which is set to scan across a range of *m/z* ratios. In this way, it is possible for corresponding parent and daughter ions of specified *m/z* to be scanned through to the detector [147].

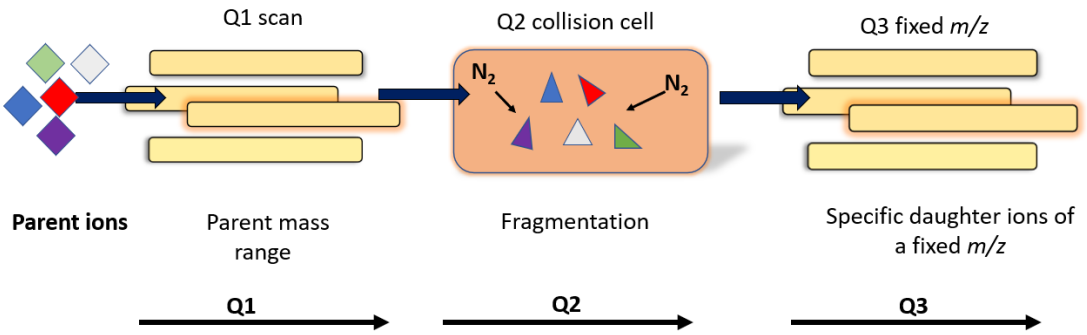


Figure 1.18 Triple quadrupole/tandem MS Parent ions of interest are selected in Q1. They are fragmented in Q2 and the daughter ions of a fixed mass charge *(m/z)* are selected and scanned in Q3. Adapted from O'Donnell et al. [147]

An LC/MS/MS method for identification and quantification of externalised PE and PS was previously developed[153]. This can be used for the measurement of externalised APL on apoptotic or other cells, for example thrombin-activated platelets. The approach is based on the use of a cell- impermeable biotinylation reagent (EZ-Link Sulfo-NHS-Biotin) used widely in proteomics for the labelling of primary amines (NH2). Using this, the externally facing headgroups of PE and PS are biotinylated on the primary amine headgroup. This method has led to the finding that human platelets externalise 5 species of PE and 3 species of PS upon agonist activation. A mass shift of 226 a.m.u. enables distinction of the biotinylated lipid from the corresponding native derivative[153].

LC/MS/MS analysis of HETEs, the oxidised metabolites of arachidonic acid is well established [225, 227]. A characteristic fragmentation pattern unique to 12-HETE can be used for the identification of oxidised phospholipids since the 12-HETE functional group is incorporated into their molecular structure. The parent ion of 12-HETE has a *m/z* of 319.2 corresponding to the $[M-H]^-$ ion which is formed by soft ionisation. Following fragmentation, the most abundant daughter ion of 12-HETE is observed at *m/z* 179.2 [227]. The six molecular species of 12-HETE-PL generated by activated platelets can be identified and quantified using HETE positional isomers and internal standards run in parallel under the same conditions.

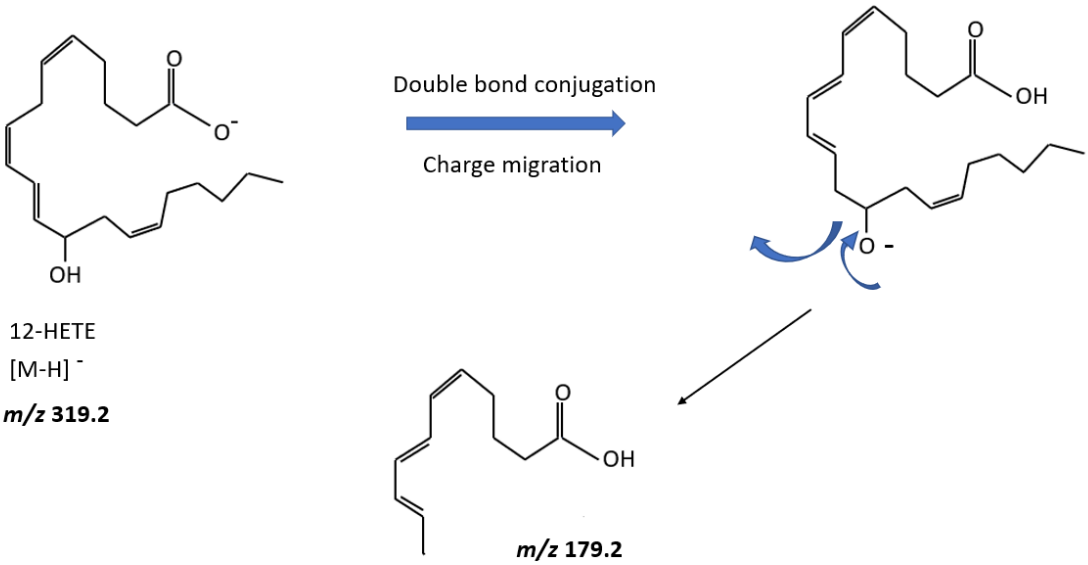


Figure 1.19 Fragmentation pattern of 12-HETE. The parent ion is produced in the ion source by 'soft ionisation'. The removal of a proton during this process generates the [M-H] - ion which has a *m/z* of 319.2. Following fragmentation in Q2, several daughter ions are produced, the most abundant of which has a *m/z* of 179.2. Adapted from Murphy et al [225]

1.10 Hypothesis and aims

Whilst it is known that the lipid composition of the platelet membrane is essential for effective coagulation, its molecular composition in health and disease has not been fully investigated. Previous research has identified novel oxidised lipids that are generated by activated human platelets and demonstrated that they promote coagulation in vitro and in vivo. Similarly, PE/PS species have been characterised that are externalised by human platelets following agonist activation. It is unknown whether platelet phospholipids and externalised phospholipids in patients with bleeding or thrombotic disorders of unknown cause differ from healthy controls, potentially contributing to the clinical phenotype. Methods of measuring platelet phospholipids using LC/MS/MS will enable their identification and quantification.

1.10.1 Hypothesis

Phospholipids that are generated and externalised by activated human platelets may differ in UBDs and acute DVT of unknown cause compared to healthy controls. It is hypothesised that increased quantities of native PL and/or higher generation of eoxPL may be found in acute thrombosis, whilst their deficiency may contribute to the bleeding phenotype of patients with UBD.

1.10.2. Aims

To investigate whether there are quantitative differences in externally facing non-oxidized PE and PS molecular species in the platelet membrane before and after activation in platelets from patients with UBD, DVT and healthy controls.

To determine whether there are differences in the enzymatic generation of 12-HETE-PLs in patient and control platelets before and after agonist activation.

To investigate the ability of platelets and microvesicles from healthy controls and patients to support coagulation ex-vivo as measured by thrombin generation.

To investigate the effect of the haemostatic agent DDAVP on the thrombin generation and phospholipid composition of the platelet membrane in patients with UBD.

Chapter 2-Materials and Methods

2.1. Study participants

Participating individuals were recruited into one of four groups:

1) UBD
2) Acute DVT
3) Healthy controls
4) UBD receiving DDAVP as a haemostatic agent prior to a minor or major surgical procedure

Informed consent was obtained from all participants and the study received approval from the Central London Medical Ethics Committee (16/LO/1168). Research and Developmental (R&D) approval was obtained from Cardiff and Vale University Health Board. The study was adopted on to the UK National Research Portfolio.

2.2 Criteria for study participation

Inclusion criteria for study participation were age 18 years or over, an established diagnosis of UBD or an acute episode of DVT. In addition, patients diagnosed with UBD who were undergoing either a minor or major surgical procedure and required haemostatic cover with DDAVP (0.3 μg/kg) were also included. Exclusion criteria were pregnancy, inability to consent to the study and treatment with non-steroidal anti-inflammatory drugs < 72 hours prior to recruitment or ingestion of aspirin or other antiplatelet medication within the preceding 10-14 days. Healthy controls were excluded if they had a bleeding disorder or a previous arterial or venous thrombotic event.

Table 2.1. Criteria for UBD

Significant bleeding history
Male: ISTH BAT > 4 Female: ISTH BAT > 6
Platelet count within range (150–400 × 10^9/L)
Normal prothrombin time (PT)/Activated partial thromboplastin time (APTT)/fibrinogen
Normal PFA-100, platelet aggregation studies and nucleotides
Normal von Willebrand screen (VWF: RiCoF, VWF: antigen)
Normal factor assays (VIII, XIII, XI and IX)

Table 2.2 Criteria for acute DVT

Criteria for acute DVT
Positive doppler ultrasound for DVT in one of the following locations within 24hrs of diagnosis: <u>Below knee (distal):</u> Peroneal vein Tibial vein Gastrocnemius Soleal <u>Above knee (proximal):</u> Popliteal vein Common femoral vein Femoral vein Superficial femoral vein Ileo-femoral vein

2.3 Demographic data

For all study participants age and gender was recorded. Additional information was collected from hospital notes and clinical computer records for patients. For UBD patients this included the International Society of Thrombosis and Haemostasis (ISTH) bleeding score, presence or absence of a family history of bleeding, response to previous haemostatic treatment, current medication and results of prior tests of haemostasis. For UBD patients undergoing a surgical intervention requiring haemostatic cover with DDAVP, the nature of the procedure and the response to treatment was also recorded. For patients with DVT, details regarding the site of thrombosis, presence of risk factors (e.g. immobility, pregnancy, surgery), family history of thrombosis, current medication and routine blood tests were obtained. The proformas used to collect data from patients with UBD and venous thrombosis are shown in Appendix 1.

2.4 Details of patient recruitment of bleeding and thrombotic disorders

Patients with UBD were already known to the Cardiff Haemophilia Centre and were diagnosed based upon the criteria described above.

Patients with acute DVT were recruited on the day of confirmation of a thrombus in a deep vein of the lower extremities by Doppler ultrasound.

2.5 Isolation of washed platelets for thrombin generation and phospholipid analysis

The following buffer solutions were prepared prior to blood draw:

1) *Acidified citrate dextrose (ACD)*

85 mM trisodium citrate, 65 mM citric acid, 100 mM glucose, dissolved in distilled water and pH adjusted to 5.0

2) *Tyrode's buffer*

134 mM sodium chloride, 12 mM sodium bicarbonate, 2.9 mM potassium chloride, 0.34 mM disodium phosphate, 1 mM magnesium chloride, 10 mM HEPES, 5 mM glucose, dissolved in distilled water and pH adjusted to 7.4

Method:

To perform the planned analyses, a total of 70 ml of blood was obtained from study participants. Blood donations were taken via venepuncture into an antecubital vein using a wide bore butterfly needle (21-gauge) attached to a syringe. To minimise the risk of platelet activation, vacutainers were not used for blood collection and the tourniquet was released upon blood draw. A constant room temperature of 20-22 ^{0}C was maintained for the isolation steps to further minimise the risk of spontaneous aggregation.

To obtain washed platelets for thrombin generation, approximately 10.1 ml of whole blood was taken into two 20 ml syringes each containing 2.4 ml of ACD (ratio of blood: ACD 8.1:1.9, v/v). The blood was separated into two 15 ml falcon tubes, followed by centrifugation at 430 x g for 10 minutes at room temperature (20-22 ^{0}C) with no brake. The platelet rich plasma (PRP) was aspirated and divided equally into 15 ml falcon tubes and centrifuged at 1640 x g with the brake mechanism off for 10 minutes at room temperature. The platelet poor plasma (PPP) was transferred into a clean falcon tube and the remaining platelet pellet was resuspended in 10 ml of calcium - free Tyrode's buffer containing ACD (9:1 v/v). The resuspended platelets and the PPP were spun again at 1,525 x g for 8 minutes at room temperature with the brake mechanism off. The supernatant was discarded, and the platelet pellet was resuspended in 1 ml of pooled PPP. The PPP was collected following the centrifugation process to generate microvesicles.

For the isolation of washed platelets for phospholipid extraction, 16.2 ml of blood was taken into three 20 ml syringes, each containing 3.8 ml of ACD. The blood containing ACD from each syringe was separated into two 15 ml falcon tubes and underwent the same centrifugation steps described above to obtain PPP and the platelet pellet was resuspended in 5 ml of Tyrode's buffer. The platelets were left to rest for up to 30 minutes before the activation process.

2.6 Platelet concentration

The platelet concentration was determined by performing a 1: 100 dilution in Tyrode's buffer/ Tryptan blue solution (445 µl Tyrode's buffer, 50 µl Trypan Blue solution, 5 µl platelets). 10 µl of the suspension was pipetted into the chamber of a Neubauer haemocytometer containing 25 grid squares. The platelet concentration was calculated by counting the total number of platelets in the 4 outer squares and the central square (each large square contains 16 smaller squares and has a total area of 1 mm^2). The following equation was applied:

Platelet concentration = [(number of platelets counted/number of squares counted) x 25]

x 100 (dilution factor) x 10^4 x volume of suspension (ml).

For the purposes of thrombin generation, washed platelets were suspended in 1 ml of pooled PPP and diluted to 1.5 x 10^8/ml with pooled PPP. For phospholipid analysis, a platelet concentration of 2 x 10^8/ml was used by diluting the 5 ml platelet suspension with further Tyrode's buffer as necessary.

2.7 Preparation of microvesicles

Micovesicles were generated from PPP collected after the centrifugation steps described above. 1 ml of PPP was collected into a 1.5 ml Eppendorf tube and centrifuged at 16,060 x g for 30 minutes. 750 µl of supernatant was removed and the sample was re-suspended up to 1 ml with Tyrode's buffer before a further centrifugation step (16,000 x g for 30 minutes). After removal of the supernatant, the resultant pellet was resuspended in 250 µl of pooled PPP and used in the thrombin generation assay.

2.8 Preparation of pooled PPP

Pooled PPP was prepared for the resuspension and/or dilution of the washed platelets and microvesicles used in the thrombin generation assay. Blood was collected from sixteen healthy volunteers, all of whom were free from anti-platelet and non-steroidal anti-inflammatory medication for 14 days. Approximately 36 ml of blood from each donor was drawn into a syringe containing sodium citrate and corn trypsin inhibitor (CTI) (Haematologic Technologic, UK) to yield a total volume of 40 ml of 0.32 % citrated blood with 0.59 U/ml of CTI activity. The blood from each syringe was transferred to 2 x 50 ml falcon tubes for each donor. The samples were centrifuged at 460 x g at room temperature with the brake mechanism turned off for 10 minutes. The resultant platelet rich plasma was collected into 2 x 15 ml falcon tubes for each donor and centrifuged at 1730 x g for 10 minutes with no brake. The plasma was pipetted into clean 2 x 15 ml falcon tubes and spun a final time at 1730 x g for 10 minutes with the same conditions applied. Finally, the plasma was mixed thoroughly in a sterile conical flask before being distributed into 1 ml aliquots and stored at – 80 ^{0}C.

2.9 Platelet activation

For lipid extraction experiments, 1 ml of washed platelets at a concentration of 2 x 10^{8}/ml in Tyrode's buffer was aliquoted into 1.5 ml Eppendorf tubes. To activated samples, 1 mM calcium chloride was added before incubation at 37 ^{0}C for 5 minutes with gentle mixing. The platelets were then activated with 0.2 units/ml thrombin (Sigma Aldrich, UK) and incubated at 37 ^{0}C for a further 30 minutes prior to lipid extraction.

2.10 Biotinylation of externalised APL and total lipids

The following reagents were prepared in advance:

1) 5 mg/mL EZ-Link Sulfo-NHS-Biotin (11 mM) (Thermo-Fisher Scientific, UK) dissolved in Tyrode's buffer.

2) 0.04 g/mL Lysine (250 mM) (Sigma-Aldrich, UK) dissolved in Tyrode's buffer.

3) 6.83 mg/mL N-Hydroxysuccinimide (NHS) biotin (20 mM) (Thermo-Fisher Scientific, UK) dissolved in DMSO.

Method:

Following thrombin activation, 200 µl of activated and unactivated platelets were separately aliquoted into 1.5 ml eppendorf tubes. For the biotinylation of externalised APL (PE and PS), 86 µl of EZ-Link sulfo-NHS biotin was added to half of the samples and left at room temperature for 10 minutes with gentle occasional inversion. 72 µl of lysine was then added as a quenching agent, and the samples were again left for a further 10 minutes. At the end of the incubation period, 42 µl of Tyrode's buffer was pipetted into each sample to give a total volume of 400 µl.

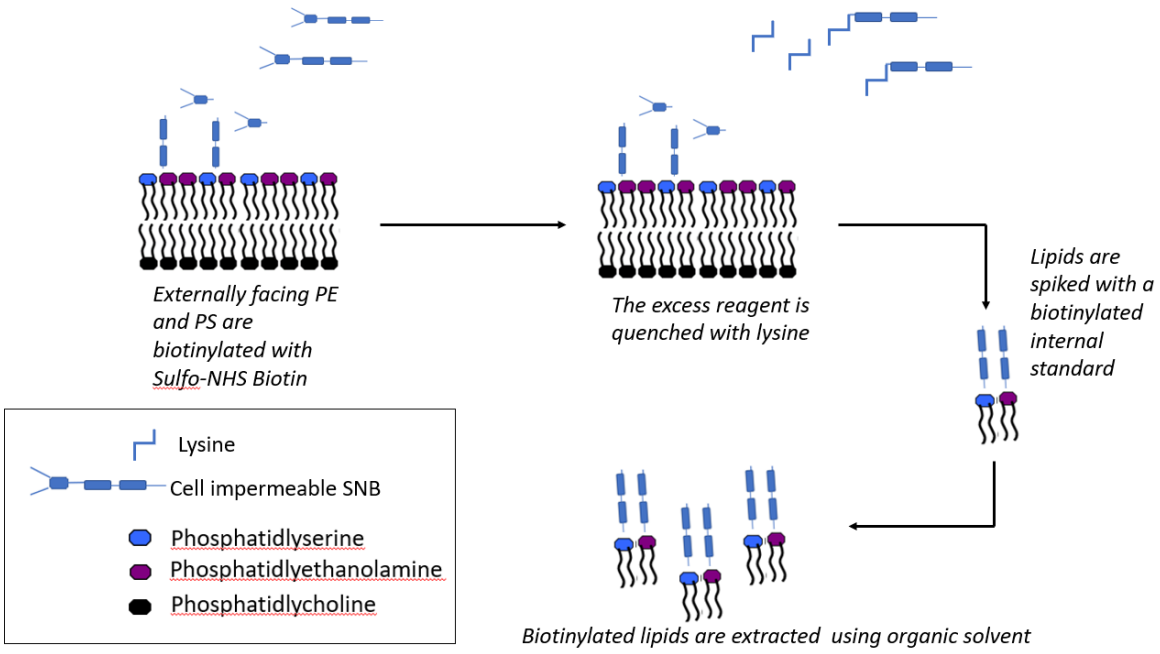


Figure 2.1 Biotinylation of externally facing APL with Sulfo-NHS-Biotin reagent. Image modified from Thomas et al [153]

To the remaining half of samples, the cell permeable analogue NHS-biotin was added for the biotinylation of total lipids. 20 µl of 20 mM NHS-Biotin was added to the samples and they were incubated at 20°C for 10 minutes. 180 µl of Tyrode's buffer was then added to achieve a final volume of 400 µl. Externalised APL could then be calculated as a fraction of the total to give a percentage.

2.11 Lipid extraction

Externalised APL and total lipids

10 ng of biotinylated standards 1,2-dimyristoyl-sn-glycero3-PS (DMPS) and 1,2-dimyristoyl-sn-glycero-3-PE (DMPE) were added to each glass extraction tube containing 1.5 ml of solvent mixture (chloroform: methanol 1:2). 400 µl of the platelet sample was transferred into each extraction tube to obtain a 1: 3.75 ratio of aqueous sample: solvent mix. The mixture was vortexed thoroughly (1 minute / sample) after which 0.5 ml of chloroform was added with further vortexing. Lastly, 0.5 ml of HPLC – grade water was added, and the samples were vortexed thoroughly. The samples were then centrifuged for 5 minutes at 500 x g at 20 - 22 ^{0}C. The lower layer was collected with a glass Pasteur pipette, taking care to avoid contamination with the upper layer by applying gentle positive pressure. Following transfer to a clean glass extraction tube the samples were dried at 30 ^{0}C in a Rapidvap evaporation system and resuspended in 100 µl of methanol prior to storage at -80 ^{0}C.

To identify biotinylated PE and PS species, lipid extracts were analysed on an AB Sciex 4000 Q-Trap mass spectrometer using an Ascentis C18 column (5 µm, 150 × 2.1 mm, Sigma-Aldrich) and an isocratic mobile phase consisting of methanol with 0.2% (wt/vol) ammonium acetate at a flow rate of 400 µl min^{-1}. These conditions were maintained for 25 minutes per sample. 20 µl of lipid extract from each sample was injected onto the HPLC column and biotinylated multiple reaction monitoring (MRM) transitions (parent to daughter ion) were monitored in negative ion mode as described in Table 2.3. Biotinylated PE was monitored by the formation of the daughter ion (Sn2 carboxylate anion). A neutral loss of 313 a.m.u. representing the biotinylated serine head-group was used to identify the biotinylated PS species of interest. The collision energies and declustering potential for each analyte are also outlined in Table 2.3.

Table 2.3 Analyte and mass transition conditions for biotinylated lipids

Biotinylated Analyte	Biotinylated MRM Transition [M-H]-	Declustering Potential (V)	Collision Energy (V)	Collision cell potential (volts)
DMPE-B (14:0/14:0-PE)	860/227	-135	-60	-13
SpAPE-B (18:0p/20:4-PE)	976/303	-160	-60	-5
SAPE-B (18:0a/20:4-PE)	992/303	-170	-58	-5
PpAPE-B (16:0p/20:4-PE)	948/303	-160	-60	-5
SOPE-B (18:0a/18:1-PE)	970/281	-170	-58	-5
OpAPE-B (18:1p/20:4-PE)	974/303	-160	-60	-5
DMPS-B (14:0/14:0-PS)	904/591	-150	-42	-17
SOPS-B (18:0a/18:1-PS)	1014/701	-140	-44	-23
DOPS-B (18:1a/18:1-PS)	1012/699	-150	-46	-23
SAPS-B (18:0a/20:4-PS	1036/723	-145	-42	-23

2.12 Synthesis of biotinylated standards

To quantify externalised APL (PE/PS), the following lipid standards were purchased from Avanti Polar Lipids:

1,2-Dimyristoyl-sn-glycero-3-PS (DMPS; 14:0/14:0-PS)

1,2-Dioleoyl-sn-glycero-3-PS (DOPS; 18:1a/18:1-PS)

1-Stearoyl-2-oleoyl-sn-glycero-3-PS (SOPS; 18:0a/18:1-PS)

1-Stearoyl-2-arachidonoyl-sn-glycero-PS (SAPS; 18:0a/20:4-PS)

1,2-Dimyristoyl-sn-glycero-3-PE (DMPE; 14:0/14:0-PE)

1-(1Z-stearoyl)-2-arachidonoyl-sn-glycero-3-PE (SpAPE; 18:0p/20:4-PE)

1-stearoyl-2-arachidonoyl-sn-glycero-3-PE (SAPE; 18:0a/20:4-PE)

1-stearoyl-2-oleoyl-sn-glycero-3-PE (SOPE; 18:0a/18:1-PE)

To synthesise the biotinylated standards, 1 mg of each lipid was added to a 1.5 ml glass vial and evaporated to dryness under a nitrogen stream. 220 µl of chloroform and 110 µl of methanol were then added and the solvent mixture vortexed. 6 mg of NHS-Biotin was weighed and transferred to the vial with further vortexing. 3.3 µl of triethylamine was added to the solvent mixture which was then incubated at room temperature for 30 minutes. The sediment of the

excess NHS-Biotin was removed following centrifugation at 500 x g for 5 minutes at 20 ^{0}C. The resultant solvent was carefully transferred to a clean glass vial and a further 220 µl of chloroform and 110 µl of methanol were added to the original vial containing the NHS biotin sediment. The solvent mixture was vortexed thoroughly and the centrifugation step repeated. The solvent fractions were finally combined and pipetted into a clean pre-weighed glass vial. The sample was then evaporated to dryness under a nitrogen stream and the vial was re-weighed. The difference was calculated and used to quantify the amount of lipid in the vial prior to resuspension in methanol. The standards were stored at -80 ^{0} C.

The biotinylated standards were purified using reverse-phase HPLC on a Discovery C18 column (25 cm x 4.6 mm, 5 µm). The mobile phase consisted of 5 mM ammonium acetate in water (mobile phase A) and 5 mM ammonium acetate in methanol (mobile phase B). The flow rate used was 1 ml/minute. Initially, 50 % of mobile phase B was increased to 100 % over 15 minutes and then maintained at 100% for 20 minutes before re-equilibration to 50 %. The UV absorbance was measured at 205 nm. The biotinylated lipid standards were dried by evaporation under a nitrogen stream, weighed and reconstituted in methanol.

2.13 Quantification of biotinylated phospholipids

To quantify each biotinylated lipid, standard curves were produced by varying the concentration of the analyte standard with the addition of a fixed amount of the internal standard. Biotinylated internal standards (DMPE-B and DMPS-B) were each diluted to a concentration of 0.02 ng/µl. The synthesised analyte standards (SpAPE-B, SAPE-B, SOPE-B, DOPS-B, SAPS-B, SOPS-B) were mixed with methanol so each had a concentration of 2 ng/µl. The analyte mix was then serially diluted as shown in Table 2.4. Finally, 100 µl of each dilution of analyte mix was added to 100 µl of internal standard. The standard curve was then analysed using LC/MS/MS.

Table 2.4 Dilution method of standard curve analytes used for biotinylated lipids

Dilution sample	Dilution method	Concentration of each analyte standard	Final concentration after adding internal standard	Amount on column (final)
1.	Undiluted analyte mixture	2 ng/µl	1 ng/µl	10 ng
2.	150 µl of (1) with 150 µl of Methanol	1 ng/µl	0.5 ng/µl	5 ng
3.	60 µl of (2) with 240 µl of Methanol	0.2 ng/µl	0.1 ng/µl	1 ng
4.	150 µl of (3) with 150 µl of Methanol	0.1 ng/µl	50 pg/µl	500 pg
5.	60 µl of (4) with 240 µl of Methanol	20 pg/µl	10 pg/µl	100 pg
6.	150 µl of (5) with 150 µl of Methanol	10 pg/µl	5 pg/µl	50 pg
7.	60 µl of (6) with 240 µl of Methanol	2 pg/µl	1 pg/µl	10 pg
8.	150 µl of (7) with 150 µl of Methanol	0.5 pg/µl	0.5 pg/µl	5 pg
9.	60 µl of (8) with 240 µl of Methanol	0.2 pg/µl	0.1 pg/µl	1 pg

Standard curves were constructed by integrating the peak area measured in counts per second (c.p.s.) for each biotinylated lipid. The peak area of each analyte was divided by the peak area of the biotinylated internal standard of the corresponding lipid species to obtain a ratio e.g. SpAPE-B /DMPE-B, DOPS-B/DMPS-B. Similarly, the ratio of analyte (A) to internal standard (IS) was calculated for nanogram (ng) amounts of lipid in each standard vial. Finally, the ratios of A:IS (cps) and A:IS (ng) were plotted against each other and a gradient was calculated for each analyte. The amount of internal standard (I_{ng}) added in all cases was 10ng. The following equation was applied to determine the nanograms of lipid in each sample:

$$A_{ng} = (A_{cps}/IS_{cps}) \times (I_{ng}/\text{gradient of the standard curve})$$

Where analyte standards were not commercially available (PpAPE and OpAPE), the standard curve of the most structurally related biotinylated lipid was used for quantification (i.e. SpAPE-B).

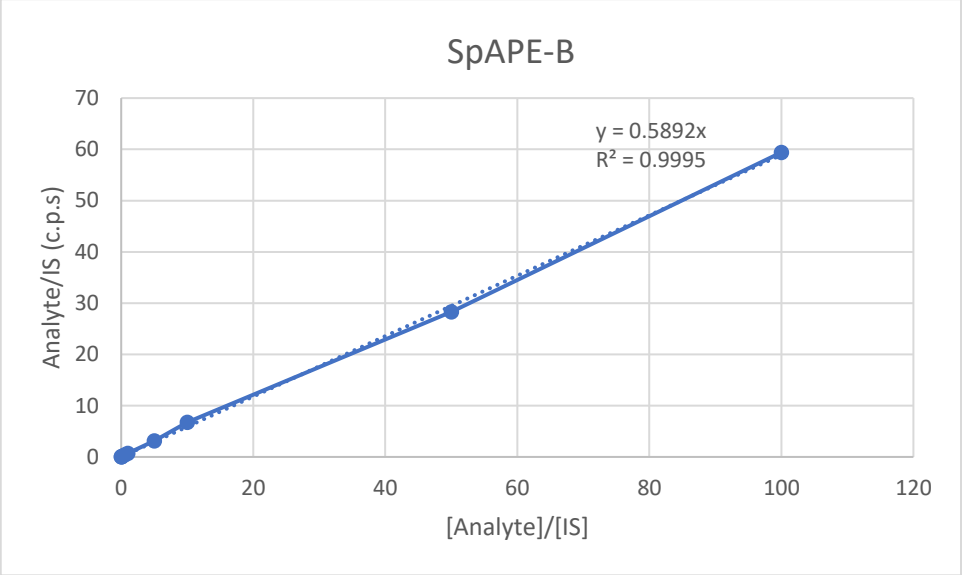


Figure 2.2. Example of a standard curve generated for quantification of the biotinylated analyte SpAPE-B. The ratio of the analyte (A): internal standard (IS) peak area in counts per second (cps) is plotted on the y axis. The concentration ratio of analyte (A): internal standard (IS) is shown on the x axis, which in this example is measured in picograms (pg). The gradient of the standard curve was 0.5892.

2.14 Lipid extraction

Esterified 12-HETE-PLs

Prior to extraction, 10 ng each of 1,2-Dimyristoyl-sn-glycero-3-PE (DMPE) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DMPC) were added to 2.5mls of extraction solvent (hexane: isopropanalol: 1M Acetic acid (30:20:2)). 1 ml of platelets at a concentration of 2 x 10^8/ml was added to each extraction tube and vortexed (1 minute/tube) followed by the addition of 2.5 ml of hexane. After further mixing, the tubes were centrifuged at 1500 rpm for 5 minutes at 4^0C. The upper layer was recovered before a further 2.5 ml of hexane was added to the original extraction tube and the extraction cycle was repeated. The resultant upper layers were combined and dried in a Rapidvap evaporation system. The samples were then resuspended in 200 µl of methanol and stored at – 80 ^{0}C prior to analysis with LC/MS/MS.

Lipid extracts were separated on an Accucore C30 2.6 µm, 100 x 3 mm column (ThermoScientific) with a gradient of 50-100 % B over 10 minutes, followed by an increase to 100 % B for 30 minutes before re-equilibration to 50:50 A: B for a further 10 minutes [A, 20 % acetonitrile: 80 % water: 5 mM ammonium acetate: 0.1 % glacial acetic acid; B, 30 % acetonitrile: 70 % isopropanol: 5 mM ammonium acetate: 0.1 % glacial acetic acid]. The flow rate used was 0.2 ml/min.

20 µl from each sample was injected onto the column and analysed by MS/MS in negative mode using MRM transitions of the parent – daughter ion (*m/z* 179.2) specific for the 12-HETE positional isomer (this is for an internal daughter ion from the 319 product ion) as specified in Table 2.5.

Table 2.5 Instrument settings and precursor to product mass transitions for oxidised PL

Analyte	MRM transition	Declustering Potential (volts)	Collision Energy (volts)	Collision cell potential (volts)
PE(18:0a_12-HETE)	782.6 → 179.2	-140	-50	-10
PC(16:0a_12-HETE)	782.6 → 179.2	-140	-50	-10
PC(18:0a_12-HETE)	810.9 → 179.2	-140	-50	-10
PE(18:0p_12-HETE)	766.6 → 179.2	-140	-50	-10
PE(18:1p_12-HETE)	764.6 →179.2	-140	-50	-10
PE(16:0p_12-HETE)	738.6 →179.2	-140	-50	-10
DMPE	634.5 → 227.2	-140	-50	-10
DMPC	662.5 → 227.2	-140	-50	-10

2.15 Quantification of eoxPL

The six molecular species of 12-HETE PL listed in Table 2.5 were quantitated using standard curves. This was generated by using a fixed quantity of an internal standard solution consisting of DMPE and DMPC, and serial dilutions of the analyte standards PE(18:0a_12-HETE) and PC(18:0_12-HETE). The oxidised lipid standards were synthesised in-house and provided by Dr Victoria Tyrell.

To prepare the internal standard mixture, DMPE and DMPC stocks were each diluted in methanol to a concentration of 0.02 ng/µl. The analyte standards PE(18:0a_12-HETE) and PC(18:0a_12-HETE) were also diluted in methanol so that each had a concentration of 2 ng/µl. The analyte mix was serially diluted as shown below. Lastly, 100 µl of analyte mix was added to 100 µl of the internal standard solution to produce the final concentration.

Table 2.6 Dilution method of standard curve analytes used for oxidised PL

Dilution sample	Dilution method	Concentration of each analyte standard	Final concentration after adding internal standard	Amount on column (final)
1.	Undiluted analyte mixture	2 ng/µl	1 ng/µl	10 ng
2.	150 µl of (1) with 150 µl of Methanol	1 ng/µl	0.5 ng/µl	5 ng
3.	60 µl of (2) with 240 µl of Methanol	0.2 ng/µl	0.1 ng/µl	1 ng
4.	150 µl of (3) with 150 µl of Methanol	0.1 ng/µl	50 pg/µl	500 pg
5.	60 µl of (4) with 240 µl of Methanol	20 pg/µl	10 pg/µl	100 pg
6.	150 µl of (5) with 150 µl of Methanol	10 pg/µl	5 pg/µl	50 pg
7.	60 µl of (6) with 240 µl of Methanol	2 pg/µl	1 pg/µl	10 pg
8.	150 µl of (7) with 150 µl of Methanol	0.5 pg/µl	0.5 pg/µl	5 pg
9.	60 µl of (8) with 240 µl of Methanol	0.2 pg/µl	0.1 pg/µl	1 pg

The standard curve was then analysed using LC/MS/MS and monitored using precursor to product transitions listed in Table 2.5. The oxidised phospholipids were quantified in the same manner as described in Section 2.13. Following integration of the peak area of each lipid, the ratio of analyte to internal standard was calculated in counts per second and then for ng amounts of lipid. A standard curve was generated from plotting the two ratios against each other such that the amount of lipid in each sample can be calculated from the gradient. A peak was only integrated if it was at least 3 times the height of the baseline noise (signal: noise ratio) otherwise it was considered below the limit of detection.

2.16 Thrombin generation using calibrated automated tomography (CAT)

All phospholipids used in the assay were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). The following buffers were prepared:

Liposome buffer.

20 mM HEPES, 100 mM NaCl, dissolved in distilled water and pH adjusted to 7.35.

Fluo-buffer.

20 mM HEPES, 0.6% BSA, 0.02 % sodium azide, dissolved in distilled water.

2.17 Generation of TF-containing liposomes

Thrombin generation was triggered by TF- bearing liposomes. The liposomes were synthesised using 65% 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 30% 1-stearoyl-2-arachidonoyl-sn-glycero-3-PE (SAPE) and 5% 1-Stearoyl-2-arachidonoyl-sn-glycero-PS (SAPS) (lipid stocks 1 mg/ml). Specifically, 27.1 µl of DSPC, 11.5 µl of SAPE and 2.09 µl of SAPS were dried in a glass vial by evaporation under nitrogen and resuspended in 460 µl of liposome buffer. 40 µl of a 25 nM stock of recombinant tissue factor (rTF) (Cambridge Biosciences, UK) was added to the mixture prior to 10 freeze thaw cycles with liquid nitrogen, with each cycle lasting up to 1 minute. Liposomes were generated by passing the mixture through a Lipsofast TM mini - extruder with 100 nm pore membranes (Avestin) nineteen times. Innovin was not used because the TF concentration and phospholipid composition is unknown. The amount of TF incorporated into the trigger liposomes was not determined.

2.18 Measurement of thrombin generation by Calibrated Automated Tomography (CAT)

Washed platelets at a concentration of 1.5 x 10^8/ml were used obtained by the methods described above (Section 2.1). To determine the ability of the platelets to support thrombin generation, 80 µl of washed platelets were added in triplicate to wells of a 96-well plate. Similarly, the washed microvesicle suspension (80 µl) was added to the plate in triplicate and as a control, 80 µl of the pooled PPP was pipetted separately into the wells of the plate. The pooled PPP used as a control for each experiment was from the same pool used to resuspend and dilute the washed platelets and microparticles.

Prior to addition of the trigger solution, a further dilution of the liposomes was made by adding 487.7 µl of liposome buffer to 12.5 µl of liposomes (2.5 µM lipid/50 pM recombinant TF). 20 µl of the diluted trigger solution was then added to each well to yield a final concentration of 0.5 µM phospholipid and a nominal final concentration of 10 pM recombinant TF. The nominal final concentration of TF quoted assumes that all TF had been incorporated into the liposomes. The actual final concentration of TF added to the assay was unknown and almost certainly less than 10 pM. The amount of liposomes used to trigger the assay was adjusted empirically to ensure that the assay was sensitive to the platelet phospholipid surface (for details see Chapter 4). For every plate that was run, a separate calibrator well containing thrombin calibrator (α-2 macroglobulin) without liposomes was used.

The plate was placed into a Fluoroskan Ascent Reader and incubated at 37 ^{0}C for 10 minutes. Thrombin generation was initiated by an automated washing step with calcium chloride and subsequently the addition of fluorogenic substrate (Z-Gly-Gly-Arg from Bachem, UK) in fluo-buffer to yield a final concentration of 5 mM fluorogenic substrate and 20 mM calcium chloride. The reaction was then allowed to proceed for 60 minutes.

2.19 Statistical analysis

For each sample, experiments were carried out in triplicate with the data point for each sample representing the mean. Mass spectrometry data was processed using MultiQuant Software for the purposes of integration of peak area. Data was analysed using Graph Pad Prism 5. The statistical significance of difference between two sets of data from separate study groups was calculated using the Mann Whitney U test. Analysis of the relationship between variables within a study group was performed using Pearson's correlation. A p value of < 0.05 was considered statistically significant.

Chapter 3- Patient demographics

3.1. Demographics and clinical data of UBD and DVT patients

A total of 82 participants were recruited into the study. There were 29 healthy controls recruited from the local population in accordance with the inclusion and exclusion criteria detailed in Chapter 2. The median age of healthy controls was 45 years (IQR 40-58). Details of age and gender of the healthy volunteers compared with UBD and thrombosis patients are shown in Table 3.1.

In the UBD cohort, a total of 21 patients were recruited, all of whom were known to the Cardiff Haemophilia Centre and had been diagnosed using the criteria outlined in Chapter 2 (Table 2.1). The median age of patients with UBD at recruitment was 66 years and there was a marked female predominance (19 females, 2 males). The median ISTH bleeding score was 10 (IQR 7-12). Bleeding following surgery, post-dental extraction and menorrhagia were the most frequent symptoms, each occurring in 76% of cases. All but 2 had previously undergone a haemostatic challenge covered with either tranexamic acid alone or in combination with DDAVP. Of the 21 UBD patients, 19 had previously participated in the BRIDGE BPD study. This had led to the identification of a genetic mutation in two patients. In one case (PL016), a single nucleotide variant was identified in the gene encoding the actin cytoskeletal regulator tropomyosin (TPM4) in megakaryocytes and platelets [228]. In the second case (PL021), a variant in the MYH9 gene was reported, although there were no associated clinical features of renal impairment, hearing loss or cataract formation. In both cases, the platelet count was within normal range.

Additionally, 2 patients with known platelet PL or prothrombin consumption defects were included in the analysis to provide direct comparison of known conditions of phospholipid dysfunction with the undetermined platelet procoagulant activity of the UBD cohort. These included a patient with Scott syndrome confirmed by absent Annexin V binding on flow cytometry and definitively by the presence of compound heterozygous TMEM16F mutations [229] and 1 patient with an abnormal prothrombin consumption index (43%) in whom Scott syndrome was excluded by normal annexin V binding. Lastly, one patient with a known platelet function defect (PFD) was included in the analysis on the effect of DDAVP on platelet phospholipids (Chapter 7). This patient was confirmed to have a reproducible absent response to epinephrine on platelet aggregometry testing after inclusion in the study. Full details including bleeding score and information regarding previous haemostatic challenges are shown in Appendix 1.

27 patients with DVT were recruited from the acute DVT service of Cardiff and Vale University Health Board using the criteria outlined in Chapter 2 (Table 3.3). The median age of the DVT patient was 65 years. All patients were screened for risk factors for thrombosis using a clinical proforma (Appendix 1). 9/27 patients (33%) had a previous thrombotic event and in 4/27 (15%) of cases, no precipitating factor was identified. Baseline blood investigations including full blood count (FBC), renal function (U&E), liver function tests, bone profile and a coagulation profile were performed at diagnosis. None of the patients had a diagnosis of antiphospholipid syndrome or a known hereditary thrombophilia. Details of the site of thrombosis and existing risk factors are detailed in Table 3.2.

Table 3.1 Demographics of healthy controls, UBD and DVT patients

	Healthy control n=29	UBD patients n=21	DVT patients n=27
Male (n)	16	2	18
Female (n)	13	19	9
Minimum age (years)	21	31	30
Maximum age (years)	69	82	79
Median age (years)	45	66	65
IQR (years)	40-58	58-75	49-70
Mean age (years)	46.9	63.7	59.4
SD	11.7	12.8	14
SE	2.3	2.8	2.7

Table 3.2 Details of the site and risk factors of thrombosis in DVT patients

Patient	Age/gender	Site of thrombosis	Risk factors	Family history of thrombosis
PL025	66 F	Left femoral DVT	• Previous DVT	Yes – sister (after lower limb surgery)
PL026	70 M	Right femoral DVT	• Previous DVT/PE • Oesophageal cancer	No
PL041	61 M	Left gastrocnemius DVT	• Trauma (fall from ladder 10 days prior)	Yes – mother (after surgery; knee replacement)
PL042	54M	Right femoral-popliteal DVT	• Previous DVT	Yes – father 2 DVT's (unknown circumstances)
PL045	73M	Left popliteal DVT	• Nil	No
PL046	79M	Left ilio-femoral DVT	• BMI>30	No
PL048	72F	Right popliteal vein DVT	• Previous DVT	No
PL049	68F	Left tibial vein DVT	• Hormone replacement therapy	Yes – sister DVT (post- partum)
PL051	45M	Left tibial vein DVT	• Previous DVT x 2 1st – unprovoked 2nd – following knee surgery	Yes – mother (related to combined oral contraceptive pill)
PL052	66F	Right peroneal DVT	• BMI> 30 • Previous DVT	No
PL053	41F	Left peroneal DVT	• Nil	No
PL054	54M	Left femoral-popliteal DVT	• Travel > 4 hours	No
PL055	70M	Left popliteal DVT	• Previous DVT (following leg fracture)	No
PL056	66M	Right popliteal DVT	• Previous DVT (unprovoked)	Yes
PL059	78F	Left ilio-femoral DVT	• BMI>30 • Travel >4 hours	No
PL062	34M	Right popliteal DVT	• Nil	Yes- mother DVT (unknown circumstances)
PL064	49F	Right peroneal DVT	• Recent surgery in 12 weeks (anterior cruciate ligament repair)	No
PL065	30M	Right tibial vein DVT	• Recent trauma (fracture of fibula)	No
PL068	36M	Left femoral DVT	• Nil	No

PL069	55M	Right popliteal DVT	• Previous DVT (unprovoked)	Yes – mother DVT/PE (unknown circumstances)
PL070	46F	Right peroneal DVT	• Travel > 4 hours • Varicose veins	No
PL071	76M	Left femoral DVT	• Family history of DVT	Yes – mother DVT (unknown circumstances)
PL073	65M	Left popliteal DVT	• Travel > 4 hours	Unknown
PL077	72M	Left tibial DVT	• BMI>30 • Recent surgery in 12 weeks (manipulation of total hip replacement) • Recent inpatient stay in 12 weeks	No
PL079	49M	Left tibial DVT	• Trauma lower limb	No
PL080	68F	Left peroneal DVT	• Malignancy • Previous PE • Family history of DVT	Yes – sister DVT (post-surgery), mother DVT (post-partum)
PL081	61M	Right popliteal DVT	• Recent trauma (fracture distal fibula 1 week prior)	No

3.2 Sample collection

There were 72 samples used for thrombin generation measurements and 66 lipid extracts available for analysis in the experiments conducted in Chapters 4, 5 and 6 of the thesis. There were 4 healthy controls who did not re-attend for all experiments to be performed and therefore there was insufficient sample available for complete analysis. In 3 DVT patients, spontaneous platelet aggregation occurred in all samples during the initial stages of the isolation process and therefore these samples could not be used in any of the experiments. In 3 other DVT patients, visible partial platelet clumping was evident in the resting stage following their isolation, in at least in half of the samples (n=3). In 2 DVT patients, fine 'grains' of platelets were visible in samples following thrombin activation and the incubation step, but aggregation had not occurred. No platelet aggregation was seen in the UBD patients and partial aggregation was seen in one healthy control. As described in Chapter 2, methods for platelet isolation were optimised in order to minimise the risk of platelet aggregation. This involved the use of a wide bore needle (21 gauge), removal of the tourniquet before the blood draw and maintaining room temperature at 20-22^{0}C. Despite the above, an increased incidence of spontaneous platelet aggregation was observed in the DVT cohort compared to healthy controls and UBD patients. In the event of complete spontaneous platelet aggregation, samples were discarded and not analysed further.

Table 3.3 Details of samples available for analysis

Cohort	Number of samples available for analysis		Spontaneous Platelet aggregation		Non-returners (within 48 hours)
	TG	PL	Partial	Complete	
Healthy controls n=29	27	24	1	0	4
UBD patients n =20	20	20	0	0	0
Scott syndrome n=1	1	1	0	0	0
Abnormal PCI n=1	1	1	0	0	0
DVT patients n=27	23	20	3	3	1

(TG; thrombin generation PL; phospholipid, partial platelet aggregation; affecting either TG or PL samples, complete platelet aggregation; affecting both TG and PL samples; UBD = Unclassified bleeding disorder; DVT= Deep vein thrombosis; PCI = prothrombin consumption index)

Chapter 4 - The ability of platelet and microvesicle phospholipid surfaces to support thrombin generation in patients with UBD, DVT and healthy controls

4.1 Introduction

The experiments detailed in this chapter sought to investigate the ability of healthy control and patient platelets and microvesicles to provide a phospholipid surface to support coagulation using the thrombin generation assay. The same pooled plasma was used for all tests so that variation in pro- and anticoagulant factors did not affect the results. The assays were tailored so that thrombin generation was dependent on the phospholipid surface supplied by the platelets or microvesicles. This assay was used to compare individuals with an acute thrombosis and unexplained bleeding disorders with healthy controls.

Commercial trigger solutions that are available to initiate coagulation in the thrombin generation assay such as "Stago PPP low" and "Stago PPP high" have TF concentrations of 1 pM and 5pM respectively, however the exact composition and concentration of phospholipid is unspecified. Similarly, the phospholipid and TF composition of Innovin is unknown. This means that, although the amount of TF protein is known, the specific activity of TF will vary because this is crucially dependent on the phospholipid membrane in which the tissue factor is reconstituted. Furthermore, in most thrombin generation assays an excess of PE/PS is added making the assay insensitive to platelet-derived phospholipid. In order to investigate the ability of phospholipids in the membranes of platelets or microvesicles to support procoagulant enzyme complexes, the trigger solution used in the experiments described here was standardised using liposomes composed of a relatively low concentration of lipids (65% DSPC, 30% SAPE, 5% SAPS) and a fixed concentration of recombinant tissue factor (rTF). The nominal final concentration of rTF in the liposomes was 10 pM, however, this assumes that all the rTF has been incorporated. Although 10 pM TF would usually be considered to be high in a standard thrombin generation assay, the activity of the TF cannot be compared to Stago reagents or Innovin because the phospholipid environment is different. The trigger liposomes were adjusted so that a background amount of FXa and thrombin would be produced in the pooled plasma activating FVIIIa and FVa before the Xa was inactivated by TFPI, as shown in Figure 4.1. The FVIIIa and FVa then allowed additional thrombin to be generated through the tenase and prothrombinase compexes supported by phospholipids on the surface of washed platelets and microvesicles. The contribution of the phospholipid surfaces could be calculated by subtracting the thrombin generated by the control liposomes alone.

As shown in Figure 4.1, sequential lowering of the lipid concentration in the rTF- bearing liposome with the same nominal TF concentration resulted in increased thrombin generation. This pattern was observed when the liposome was used to trigger coagulation in PPP. Whilst this observation seems paradoxical, this was demonstrated on three separate occasions. Possible explanations could be that the reduced surface area conferred by lowering the phospholipid content of liposomes may increase the likelihood of coagulation factors binding in a more concentrated manner. In addition, reducing the phospholipid whilst maintaining the same TF concentration, would increase the number of TF molecules relative to phospholipid leading to a higher density of TF molecules in the membrane. It is possible that this combined effect was not as apparent when the control liposome was used to trigger coagulation in PRP due to the additional lipid provided by the platelets.

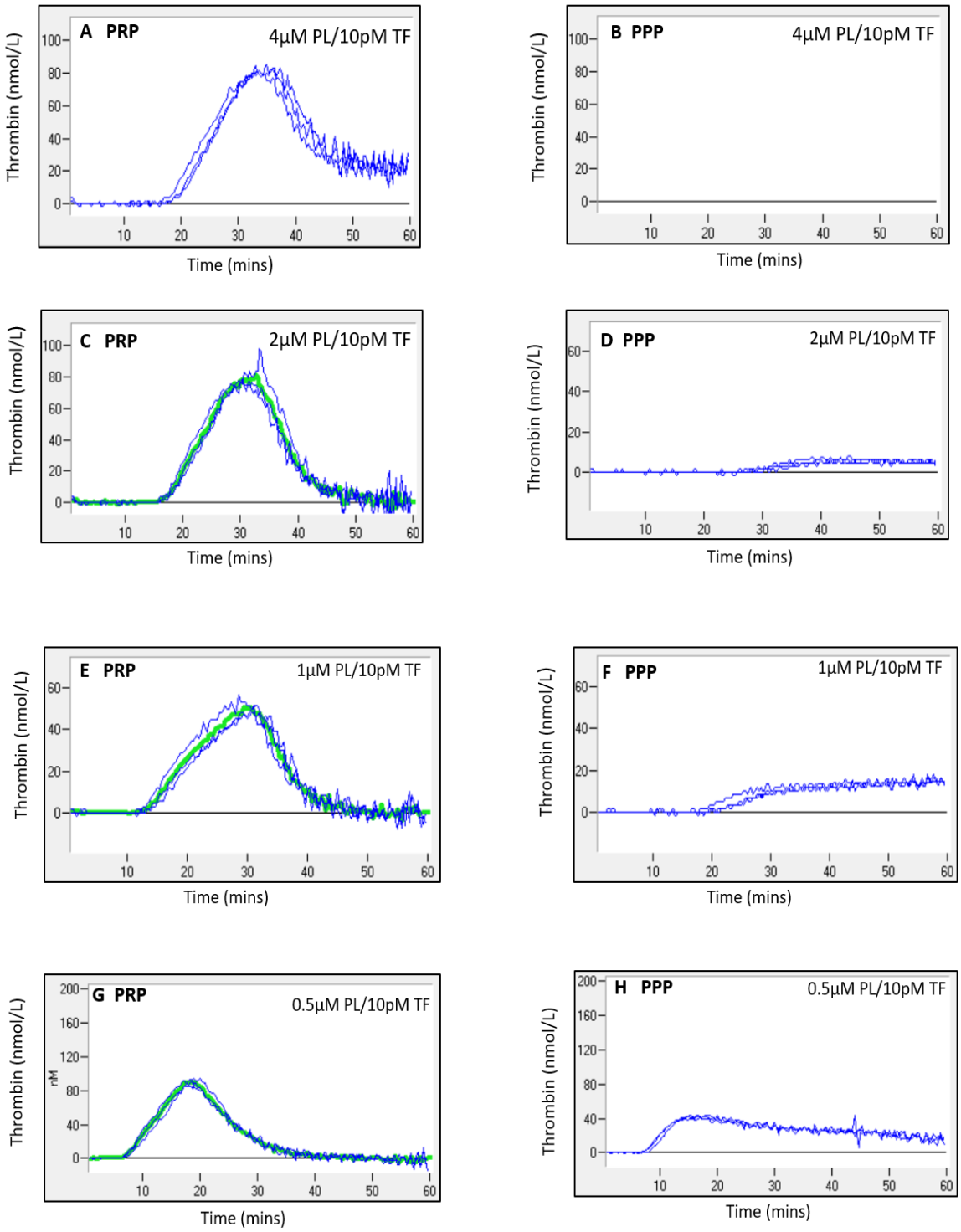


Figure 4.1. Lowering the phospholipid concentration in the rTF - bearing liposome results in increased thrombin generation in PPP (A-B) 4 µM lipid 10 pM rTF in PRP and PPP (C-D) 2 µM lipid 10 pM rTF in PRP and PPP (E-F) 1 µM lipid 10 pM rTF in PRP and PPP (G-H) 0.5 µM lipid 10 pM rTF in PRP and PPP.

Using the control liposome to activate coagulation, a series of dilutions of washed platelets from a healthy volunteer were resuspended in PPP as shown in Figure 4.2. Previous studies report a saturating effect with platelet concentrations above 2 x 10 ^{8}/ml, which could render the assay insensitive to platelet-derived phospholipid [210]. A platelet count of 1.5 x 10 ^{8}/ml was therefore used for the thrombin generation experiments performed in this thesis. To provide the same conditions of coagulation factors and inhibitors, the same pooled PPP taken into citrate and CTI was used to resuspend washed platelets and the microvesicle pellet in all experiments.

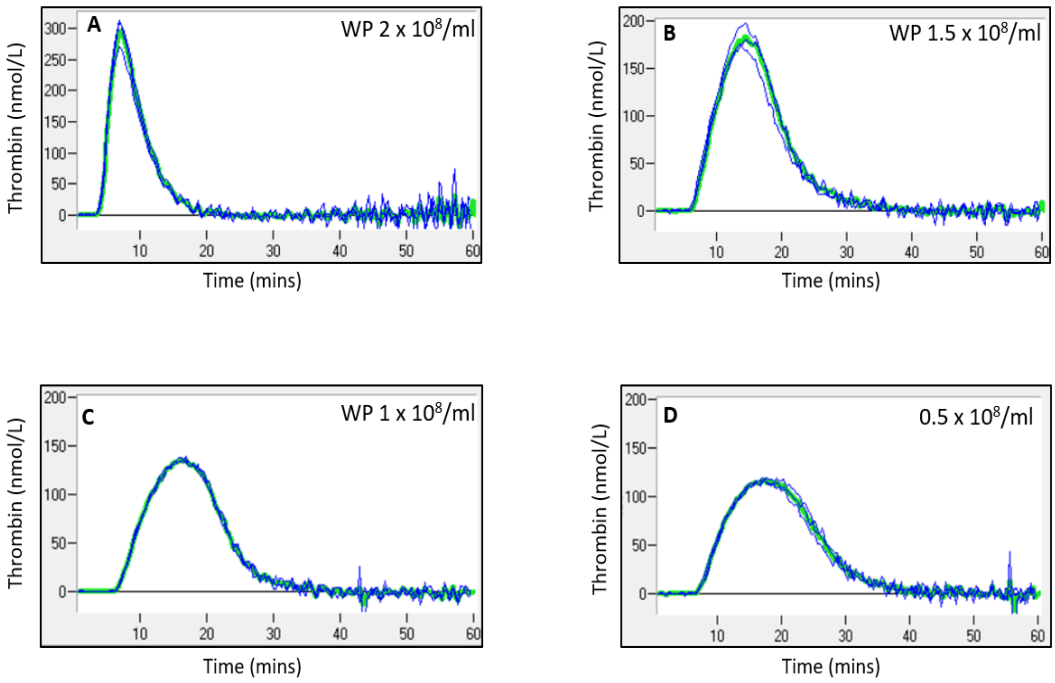


Figure 4.2 Dilutions of washed platelets from a healthy control in pooled PPP using a control liposome. The trigger used in all dilutions was the same control liposome (0.5 µM lipid, 10 pM rTF). The washed platelets were sequentially diluted as follows: (A) 2 x 10^{8}/ml (B) 1.5 x 10^{8}/ml (C) 1.0 x 10^{8}/ml (D) 0.5 x 10^{8}/ml.

For each experiment performed, washed platelets were diluted to 1.5 x 10^8/ml and the standard trigger liposome was utilised (Figure 4.3). This was used for the calculation of each parameter for both washed platelets and microvesicles. An example from a healthy control is shown below.

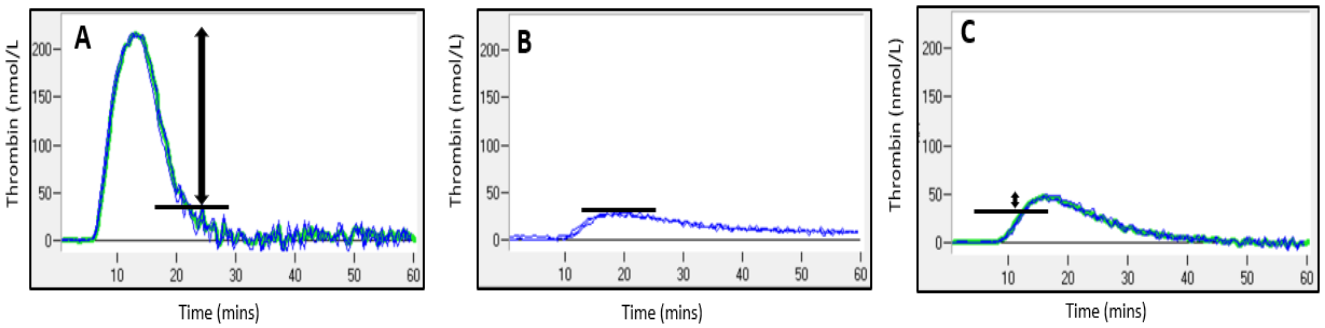


Figure 4.3 Example of measuring peak thrombin using washed platelets and microvesicles from a healthy control. (A) Thrombin generation supported by washed platelets activated by a control liposome in standardised PPP (B) Thrombin generation measured in a control liposome in standardised PPP (C) Thrombin generation supported by microvesicles activated by a control liposome in standardised PPP. The control liposome (B) generates a measurable amount of thrombin shown by the horizontal black line. The vertical black arrows represent the additional amount of thrombin generated by washed platelets (A) and microvesicles (C) respectively which was subtracted from the background amount of thrombin measured from the control liposome (B). This principle was applied to each parameter which results in the lag time being reported as negative seconds.

4.2 Results

4.2.1 Thrombin generation supported by washed platelets in UBD patients

Lower peak thrombin generation was observed in the UBD cohort compared to healthy controls (P=0.03) although there was substantial overlap between the groups. There was no statistically significant difference observed in the ETP, velocity index and lag time between UBD patients and healthy controls (Figure 4.4). The results of each measured parameter are detailed in Table 4.1. As expected, washed platelets from the single patient with Scott syndrome generated only a minimal amount of thrombin with similar negligible changes observed in velocity index. A significant reduction in ETP was demonstrated in comparison with healthy controls and UBD patients. Addition of Scott platelets to the control liposome did not result in a shortening of the lag time. The finding that Scott syndrome platelets were unable to support thrombin generation confirms that the assay is sensitive to reduction of externalised procoagulant phospholipids.

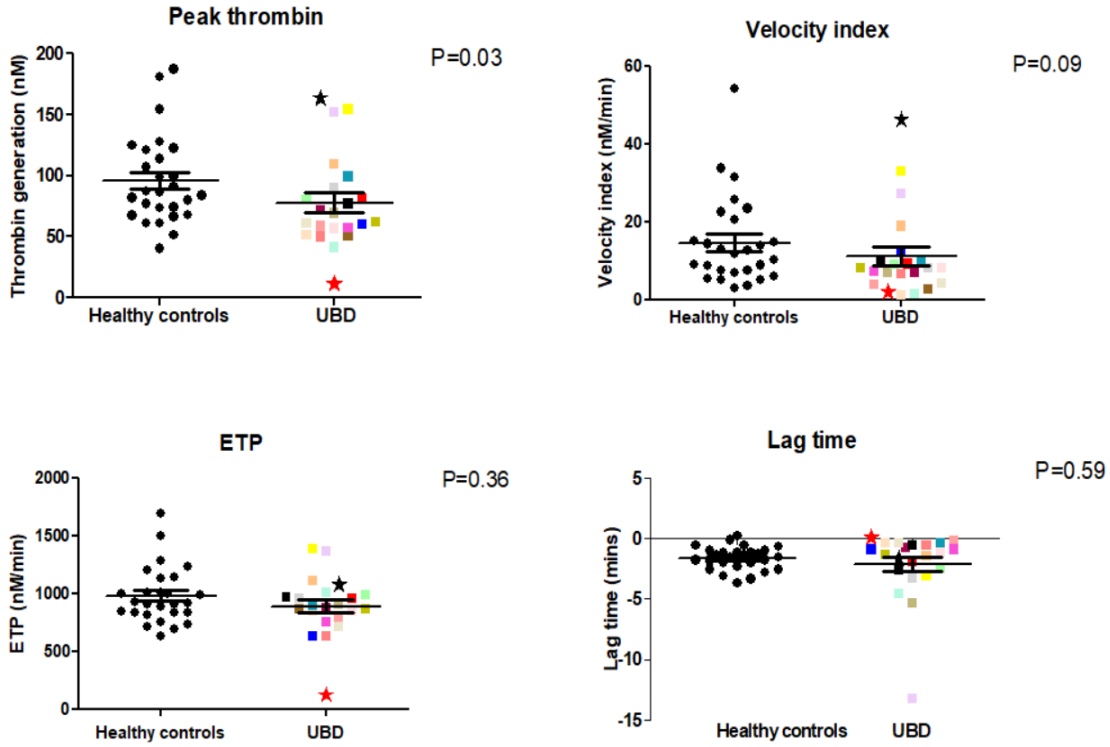


Figure 4.4 Measurements of peak thrombin are lower in UBD patients. The peak thrombin (A), velocity index (B), ETP (C) and lag time (D) measured from washed platelets in healthy controls and UBD patients is shown above. Thrombin generation parameters are shown for healthy controls (black dots) and UBD patients (coloured dots with individuals labelled consistently through the panels). Data from a patient with Scott syndrome (red star) and abnormal prothrombin consumption index (black star) is shown. Data shown are mean values (long horizontal black bar) and the standard error of the mean (short horizontal black bars). P values were calculated using the Mann Whitney U test excluding the Scott syndrome and prothrombin consumption index patients.

Table 4.1 Descriptive data for each parameter of the thrombin generation assay are shown for healthy controls and patients with UBD.

Peak thrombin generation (nM)	**Healthy controls (n=27)**	**UBD patients (n=20)**
Median	86.16	65.91
25% percentile	68.04	56.93
75% percentile	121.3	87.68
Mean	96.00	76.82
Standard deviation	36.65	31.45
Standard error	7.054	7.032

Velocity index (nM/min)	**Healthy controls (n=27)**	**UBD patients (n=20)**
Median	11.63	8.02
25% percentile	6.820	4.73
75% percentile	20.61	10.09
Mean	14.60	9.73
Standard deviation	11.47	8.08
Standard error	2.208	1.81

ETP (nM/min)	**Healthy controls (n=27)**	**UBD patients (n=20)**
Median	923.0	889.5
25% percentile	831.9	806.0
75% percentile	1134	984.0
Mean	980.8	919.7
Standard deviation	244.4	198.7
Standard error	47.04	44.42

Lag time (mins)	**Healthy controls (n=27)**	**UBD patients (n=20)**
Median	-1.560	-1.22
25% percentile	-2.330	-2.97
75% percentile	-1.000	-0.55
Mean	-1.621	-2.25
Standard deviation	0.9632	2.96
Standard error	0.1854	0.66

4.2.2 Thrombin generation supported by washed platelets in DVT patients

An increase in peak thrombin, velocity index and ETP was observed in DVT patients compared to healthy controls, although there was no significant difference in lag time. The peak thrombin and velocity index were notably higher in 4 patients within the thrombotic cohort suggesting that, in at least some patients, platelets may contribute to a prothrombotic phenotype at the time of a DVT. There was no visible evidence of spontaneous platelet aggregation in these 4 patients at the time of testing.

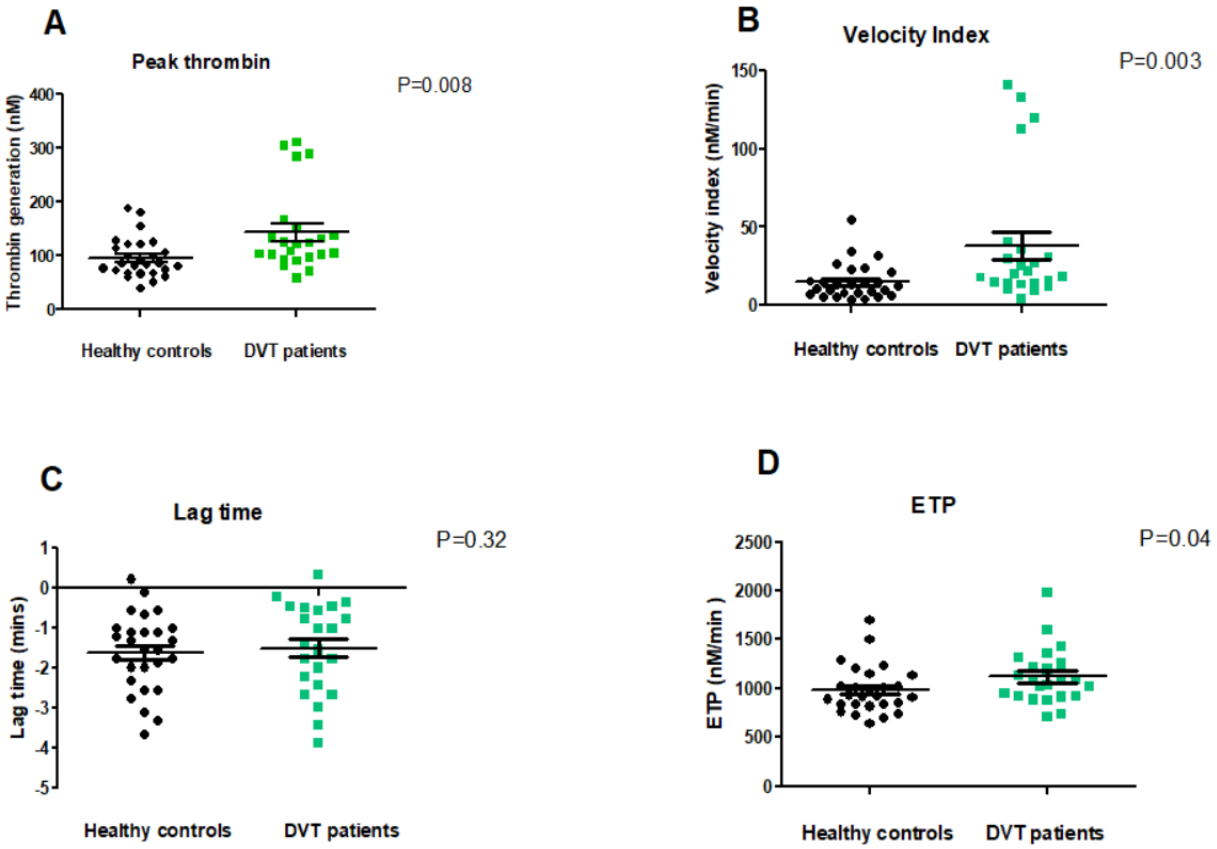


Figure 4.5 Measurements of peak thrombin, velocity index and ETP are increased in DVT patients. The peak thrombin (A), velocity index (B), ETP (C) and lag time (D) measured from washed platelets in healthy controls and DVT patients is shown above. Healthy controls and DVT patients are represented by black dots and green squares respectively. Data shown are mean values (long horizontal black bar) and the standard error of the mean (short horizontal black bars). P values were calculated using the Mann Whitney U test.

Table 4.2 Descriptive data for each parameter of the thrombin generation assay are shown for healthy controls and patients with DVT.

Thrombin generation (nM)	**Healthy controls (n=27)**	**DVT patients (n=23)**
Median	86.16	122.2
25% percentile	68.04	97.02
75% percentile	121.3	151.1
Mean	96.00	143.5
Standard deviation	36.65	76.54
Standard error	7.054	15.96

Velocity index (nM/min)	**Healthy controls (n=27)**	**DVT patients (n=23)**
Median	11.63	20.05
25% percentile	6.820	13.39
75% percentile	20.61	35.70
Mean	14.60	38.22
Standard deviation	11.47	42.64
Standard error	2.208	8.891

Lag time (mins)	**Healthy controls (n=27)**	**DVT patients (n=23)**
Median	-3.670	-3.890
25% percentile	-2.330	-2.220
75% percentile	-1.000	-0.4800
Mean	-1.621	-1.429
Standard deviation	0.9632	1.079
Standard error	0.1854	0.2476

ETP (nM/min)	**Healthy controls (n=27)**	**DVT patients (n=23)**
Median	923.0	1086
25% percentile	831.9	920.0
75% percentile	1134	1355
Mean	980.8	1157
Standard deviation	244.4	307.9
Standard error	47.04	64.21

4.3.1 Thrombin generation supported by microvesicles in UBD patients

As observed with washed platelets, peak thrombin generation supported by washed microvesicles was significantly lower in UBD patients compared with healthy controls (P = 0.01). As seen with washed platelets there was substantial overlap between the UBD patients and healthy controls (Figure 4.6). Similarly, an overall reduction in velocity index was demonstrated in the bleeding cohort although no difference in lag time or ETP was observed. The microvesicle pellet suspension obtained from the single patient with Scott syndrome generated only small amounts of thrombin as expected, although this was comparable with the peak thrombin concentrations measured in other UBD patients and some controls.

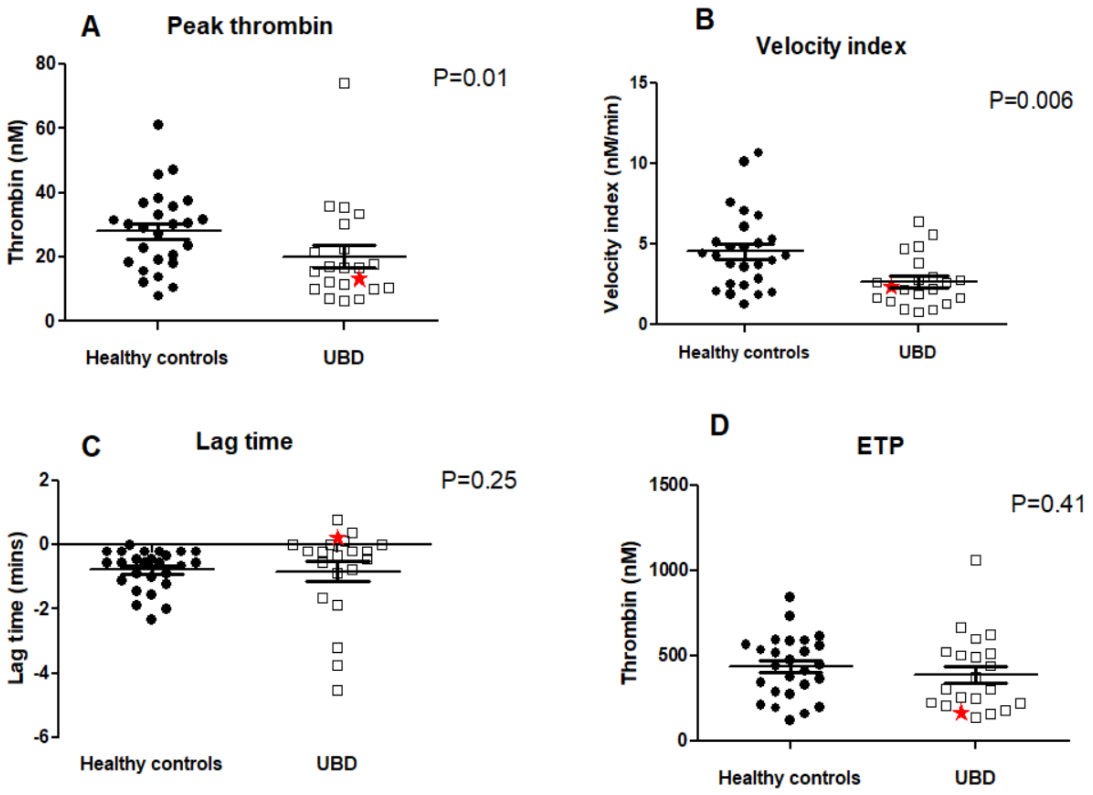


Figure 4.6 Measurements of peak thrombin and velocity index are lower in microvesicles isolated from UBD patients. Peak thrombin (A), velocity index (B), ETP (C) and lag time (D) measured from microvesicles in healthy controls and UBD patients. Thrombin generation parameters are shown for both healthy controls (black dots) and UBD patients (white squares). Data from a patient with Scott syndrome is shown (red star). Data shown are mean values (long horizontal black bar) and the standard error of the mean (short horizontal black bars). P values were calculated using the Mann Whitney U test excluding the Scott patient.

4.3.2 Thrombin generation supported by microvesicles in DVT patients

In contrast, the peak thrombin generated by washed microvesicles of DVT patients showed no difference when compared with measurements from healthy controls. The velocity index, lag time and ETP parameters were similar in the thrombotic group and the healthy controls.

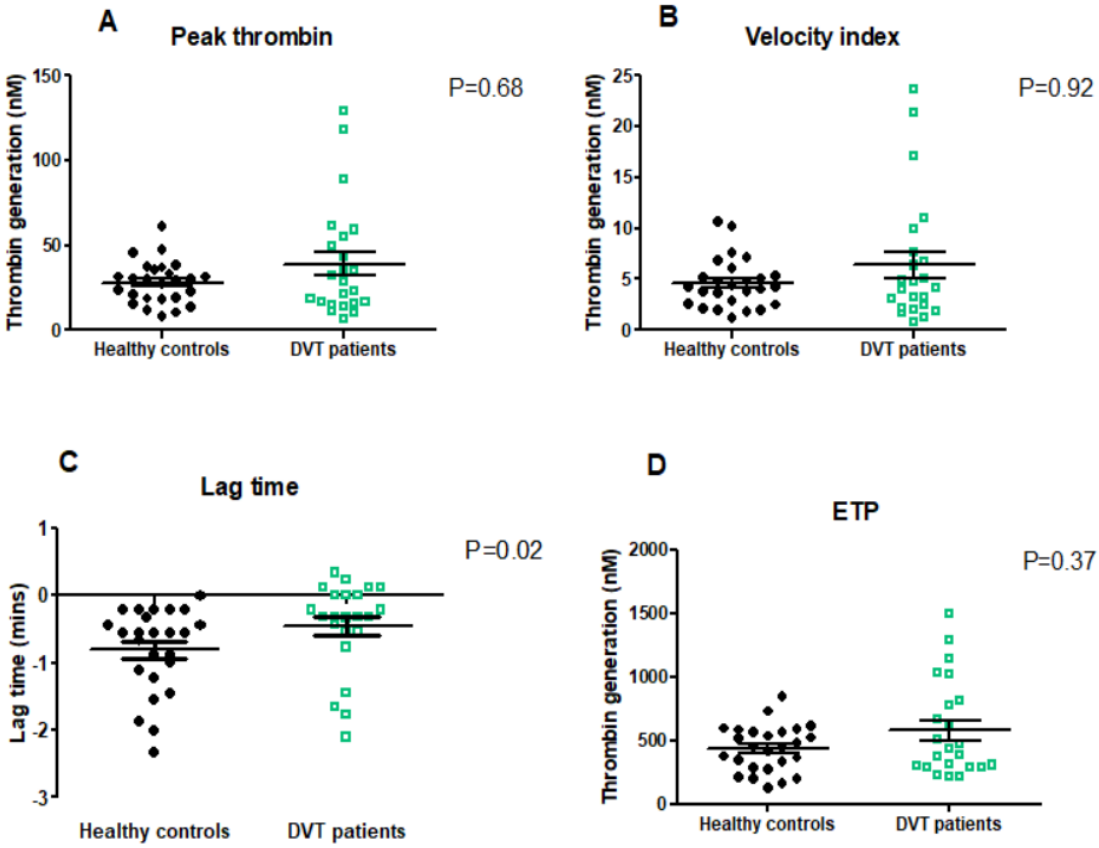


Figure 4.7 The ability of microvesicles from DVT patients and healthy controls to support thrombin generation is similar. The peak thrombin (A), velocity index (B), ETP (C) and lag time (D) measured from microvesicles in healthy controls and DVT patients. Thrombin generation parameters are shown for both healthy controls (black dots) and DVT patients (green squares). Data shown are mean values (long horizontal black bar) and the standard error of the mean (short horizontal black bars). P values were calculated using the Mann Whitney U test.

To investigate whether the peak thrombin generation supported by washed platelets was associated with the peak thrombin generation supported by microvesicles in healthy controls, UBD patients and the DVT cohort, scatter plots were constructed (Figure 4.8). No relationship between these parameters was observed in the healthy controls or the in the disease groups, however the same clustering pattern was seen in the 4 outlying DVT patients. These results suggest that the ability of an individuals' washed platelets to support thrombin generation does not parallel that of their microvesicles, either in the resting state or at the time of acute thrombosis. It is important to note that the microvesicles used in the thrombin generation assay were not purely derived from platelets, but also from other cells including neutrophils

and monocytes some of which may have been expressing TF. This may add a degree of complexity to the association and in part, account for the lack of correlation observed between the two parameters.

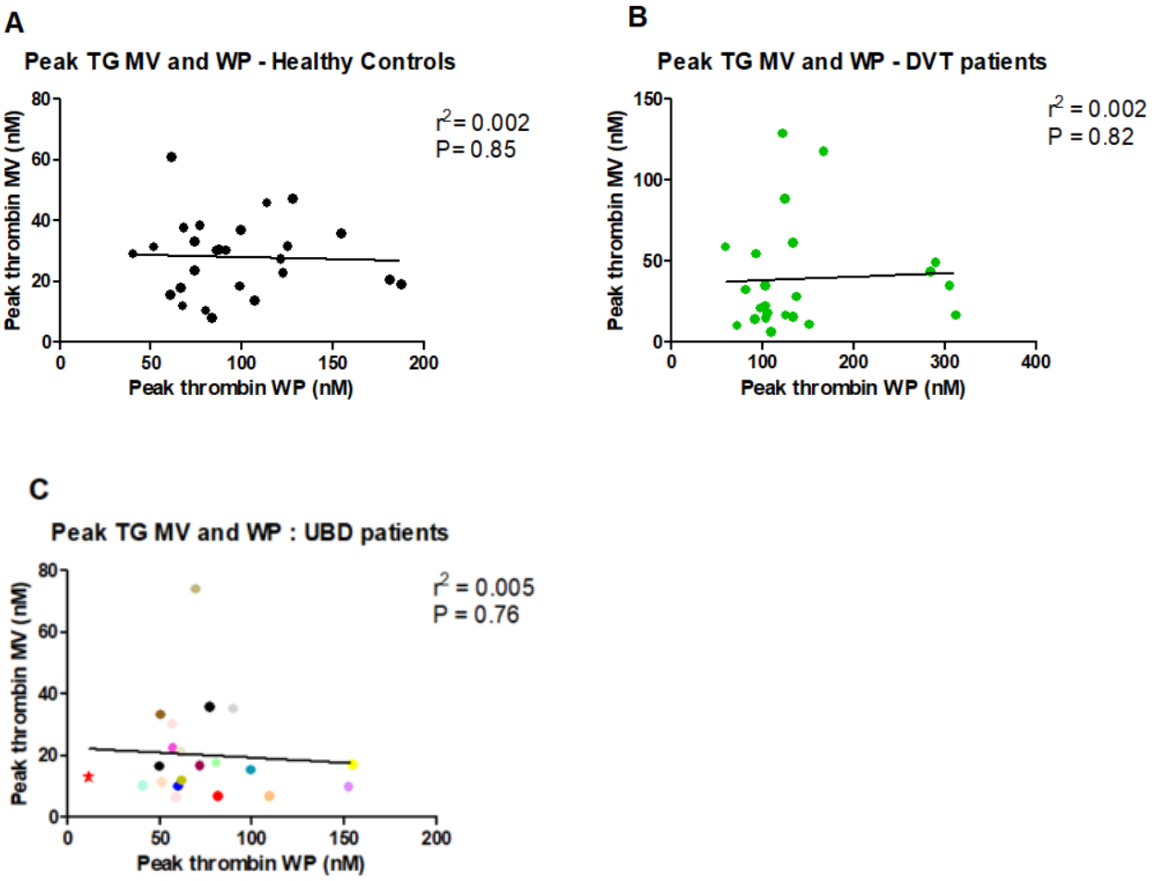


Figure 4.8 Correlation of peak thrombin measurements in washed platelets and microvesicles. The correlation of peak thrombin generation supported by washed platelets with the corresponding value of peak thrombin generation thrombin generation supported by the individuals' microvesicles is shown for healthy controls (A), DVT patient (B) and UBD patients (C). For each scatter plot, the correlation co-efficient (r squared) with the corresponding p-values (P) is shown. The Scott syndrome patient has been excluded from the analysis.

4.4 Discussion

The aim of this chapter was to investigate the ability of patient and control platelets to support coagulation measured by a thrombin generation assay adjusted to be sensitive to the contribution of phospholipid membranes. Using the methods outlined in Chapter 2, the peak thrombin and velocity index were associated with changes in both the UBD and the thrombosis cohorts compared to the controls. Both these parameters were associated with statistically significant differences in the thrombotic group, with higher levels of peak thrombin concentration ($p = 0.008$) and velocity index ($p=0.003$). Peak thrombin generation was significantly reduced in the bleeding cohort ($P=0.03$), whilst a trend towards lower velocity index ($P=0.09$) was observed in UBD patients compared with the control group. In addition, the microvesicle fraction of patients with UBDs supported lower peak thrombin and velocity index compared to healthy controls.

These results suggest that the ability of the platelet surface to support coagulation may play a role in UBDs and venous thrombosis. In the patients with excess bleeding, the ability of both their platelets and microvesicles to support global coagulation was reduced suggesting a potential novel mechanism for their bleeding. More patients will need to be investigated to establish whether the trend towards a decreased velocity index is a genuine finding. The platelets were investigated in the non-bleeding state and so reflect the normal baseline state in these patients. The ability of platelets to support coagulation is critical to normal haemostasis in vivo but is not assessed in any standard haemostatic assay and so would not have been seen in this cohort during routine clinical investigation. Whether these changes are a contributory cause of the excess bleeding in these patients will require further work and the study needs to be repeated in an independent cohort to assess whether the findings are reproducible.

The increased ability of the platelets of patients with venous thrombosis to support coagulation may play a role in the development of a thrombus. This enhanced ability to support global coagulation may interact with increased levels of coagulation factors known to predispose to thrombosis, such as FVIII and FII, or with clotting factors resistant to inactivation such as FV Leiden to induce a thrombus. FII, FVa and FVIIIa function and Protein C activation all require phospholipid surfaces and it is possible that the environment available to these coagulation factors influences their role in disease states. The samples from UBDs were taken in a non-bleeding state and so represent the baseline state of these patients. However, it is important to recognise that the samples from the DVT cohort were taken at the time of a thrombotic event and changes may have been a consequence of that event rather than causative.

There were 4 DVT patients whose platelets supported substantially higher amounts of thrombin generation compared to other patients in the cohort and controls. Although these 4 patients did not have a higher thrombotic disease burden (site and extent) at the time of testing, all of them had been previously diagnosed with one or more episodes of unprovoked VTE and therefore had a pre-existing prothrombotic tendency. A total of 9 patients had previous VTE as a risk factor, and therefore the association with this risk factor does not fully explain the higher thrombin generation observed in these 4 outliers. Overall, there was a higher incidence of spontaneous platelet aggregation in DVT patients, which has also been reported in other studies [146]. It is possible that there was a higher degree of platelet hyper-

reactivity in these four samples, although there was no macroscopic evidence of platelet aggregation. Alternatively, other pro-coagulant factors and pathways other than the thrombin-generating capacity of the platelet surface membrane may have been influential in promoting the pro-thrombotic process in these patients.

In other studies, ETP is considered to be one of the most useful thrombin generation parameters in demonstrating associations with a thrombotic or bleeding phenotype due to changes in the balance of pro- and anti-coagulant factor [221, 230]. It was anticipated that the ETP would not differ significantly amongst participants in this study because patient and control washed platelets were resuspended in the same plasma pool. Therefore, the same amount of prothrombin was in each assay potentially normalising the ETP. The same pooled plasma was used to remove the wide variability of different donor coagulation factors and plasma anticoagulants, thus increasing the sensitivity of the assay to interactions of coagulation factors with the platelet surface membrane. The TF/phospholipid vesicles were designed to initiate coagulation by generating a small amount of thrombin but provide minimal support for the propagation phase and limited support for the prothrombinase complex. TFPI was expected to inactivate further TF/FVIIa-dependent thrombin generation caused by the liposomes alone. The washed platelets or microvesicles in the assay were intended to provide support for the intrinsic tenase and prothrombinase complexes. This was successfully achieved as shown in Figure 4.1. It is therefore not surprising that the assay was unable to detect any changes in the lag time.

In the Cambridge study, 124 UBD patients were assessed with a thrombin generation assay to determine whether any diagnostic pattern was apparent. A prolonged lag time was observed in 26% of patients and a decreased ETP was reported in 19% and therefore not consistently demonstrated in the UBD cohort [185]. The thrombin generation assay used in the Cambridge study however, was performed in PPP and designed to be sensitive to coagulation factors in the plasma rather than to the platelet phospholipid surface. A phospholipid concentration of 4 μM was used rather than the minimal concentration of phospholipid used in the experiments described in this thesis.

The ability of microvesicles from healthy controls, UBD and thrombotic patients to support coagulation in standardised pooled plasma using the thrombin generation assay was also investigated. The ability of microvesicle phospholipid to support thrombin generation as shown by the parameters, peak thrombin generation and velocity index, was significantly lower in the UBD cohort compared to healthy controls suggesting a possible mechanism for the increased risk of bleeding in these patients. This is in addition to the decreased ability of platelet phospholipids from these people to support peak thrombin generation. The number of microvesicles in the supernatant of each subject was not assessed. It is not known whether the reduced thrombin generation in the UBD group was related to fewer microvesicles being present or differences in the ability of microvesicles to support coagulation. There was no difference in the ability of microvesicles in DVT patients to support thrombin generation despite the observed increase in thrombin generation associated with their platelets. This suggests that microvesicles are unlikely to play a major role in supporting enhanced activation of coagulation in this patient group.

It is important to note that the microvesicle pellet used in the thrombin generation assay was generated from resident microvesicles in the PPP supernatant of each patient. These microvesicles were formed in vivo and so reflect the innate ability of that individuals microvesicles to support coagulation. Experiments were not performed using microvesicles derived from agonist-activated washed platelets in vitro. It is possible that different results

would have been found using platelet-derived microvesicles formed from activation with a platelet agonist such as thrombin or collagen in-vitro but these would have had less physiological relevance. Although high-speed centrifugation is a recognised method for isolating microvesicles [231] there has been increasing recognition that the pellet obtained by this means may contain non-vesicular components. In recent years this has led to increasing use of size exclusion chromatography as a means of selectively isolating vesicles from platelet-free supernatant of platelet concentrates [232]. However, the unmanipulated extract used here is what is in the patient and hence may be considered of potential physiological relevance.

In summary, the ability of washed platelets to support thrombin generation in patients with UBD may contribute to the increased risk of bleeding observed in these patients. The role of platelets in venous thrombosis is less clear and the increased ability of washed platelets to support global haemostasis may be clinically significant or a consequence of the thrombotic event. Microvesicles of UBD patients supported lower amounts of thrombin but this was not seen in patients with DVT. If reproducible in an independent cohort of UBD patients, these findings may have implications for the cause of bleeding in UBD and potentially for identifying patients at risk of these disorders.

Chapter 5 - Native platelet aminophospholipids in UBD and DVT patients

5.1. Introduction

The composition of the platelet phospholipid membrane is important in regulating coagulation reactions and so the changes in thrombin generation described in Chapter 4 could be a consequence of differences in externalised APL and this question is addressed in the following chapter.

In the resting state PC, an inert phospholipid is distributed predominantly in the external leaflet and the polar APLs, PE and PS are retained in the internal leaflet [102]. Following scramblase activation, externalisation of PE and PS occurs which allows interaction between PE and PS with the Gla domains of plasma coagulation factors as discussed in Chapter 1 [233]. This event is essential for the assembly of key enzymatic complexes which ultimately result in thrombin generation occurring in vivo.

There are very limited data on the role of specific PE/PS species of the platelet membrane in relation to bleeding and thrombotic disease. It is evident that a failure of platelets to externalise PE and PS is pathological, as exemplified by the bleeding observed in Scott syndrome [154]. It remains to be proven however, whether less marked quantitative reductions in PE/PS exposure occur in uncharacterised bleeding disorders, or in contrast whether significant increases in externalised APL are contributory to the pathogenesis of venous thrombosis. Increased PS exposure has recently been reported in circulating leucocytes and platelets from patients with essential thrombocythaemia (ET) who were positive for the JAK 2 mutation and therefore considered be prothrombotic, although this has not been demonstrated in venous thrombosis without ET or any other acquired prothrombotic disorder [204]. Increased PE and PS- expressing microvesicles are known to be present in both arterial and venous thrombotic disorders [205, 234] which may mirror the composition of the platelet phospholipid surface however this has not been fully investigated to date.

The development of a lipidomics assay utilising a cell impermeable reagent has enabled the identification and measurement of externalised PE and PS by LC/MS/MS. Furthermore, this assay has enabled the identification of 5 species of PE and 3 species of PS exposed on the platelet surface following activation [153]. The aim of this chapter is to determine whether there are quantitative differences in externalised PE/PS in the platelets of patients with UBD and venous thrombosis. In addition, the correlation between the amount of thrombin generated with externalised PE and PS species on the surface of platelets was explored.

5.2 Methodology of the biotinylation assay

Biotin is a vitamin B7 compound, otherwise referred to as Vitamin H [235]. Since the late 1980's, it has been used mainly in the field of proteomics for the labelling of primary amine-containing molecules including cell surface proteins and antibodies [236]. In a similar way, the reagent EZ-link sulfo-NHS-biotin (SNB) can be utilised to derivatize externally facing APL due to the reaction between the NHS part of the compound and the amine (NH_2) headgroup of the phospholipid. SNB is readily soluble in water but is unable to penetrate the cell membrane and can therefore be used for the labelling of external APL species. N-hydroxysuccinimidobiotin (NHS-Biotin) however, is poorly soluble in aqueous solutions but is cell permeable on dissolving in an organic solvent such as DMSO. The biotinylation of total cellular lipids using NHS-biotin enables the percentage of externalised APL to be calculated.

The biotinylation method for measuring the amount of the individual species of PS and PE on the external leaflet of an activated or apoptotic cell was originally described by Thomas et al [153]. In contrast to native PC, PE and PS contain a primary amine headgroup which allows these phospholipids to be derivatized. Biotinylated species of externalised PE and PS are differentiated from their native forms by virtue of a mass change of + 226 a.m.u. The synthesis of biotinylated internal and primary standards of PE and PS as described in Chapter 2 is required for the detection and quantification of individual molecular species (Figure 5.1) [153].

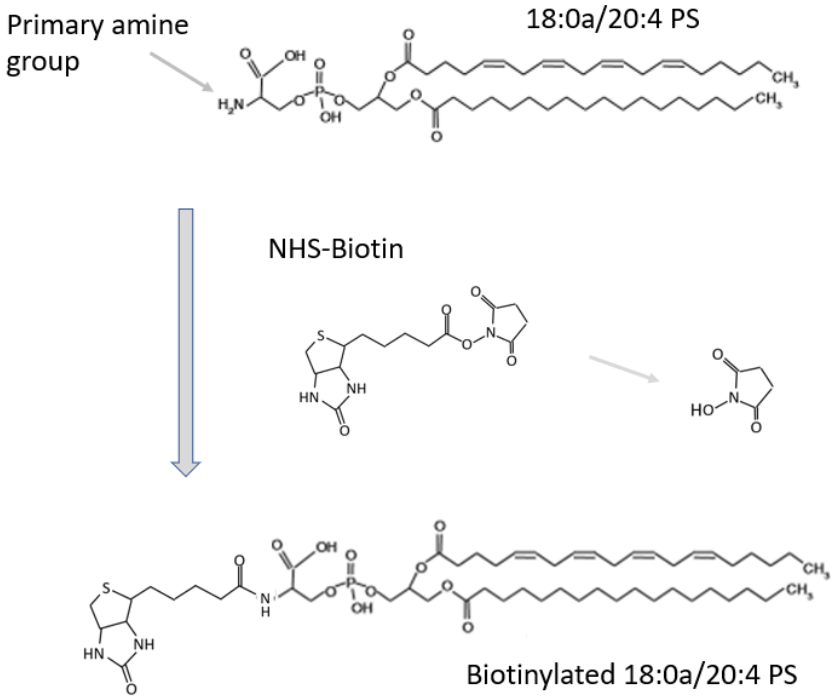


Figure 5.1 Generation of a biotinylated standard. An example of a biotinylated form of PS is shown. The biotinylated reagent (NHS-Biotin) reacts with the primary amine head-group of a phospholipid. The NHS is a by-product of the reaction. The biotinylated species of PS can be distinguished from its native form by a mass change of 226 a.m.u. Image modified from https://assets.thermofisher.com/TFSAssets/LSG/manuals/MAN0011206_EZ_NHS_Biotin_Reag_UG.pdf

The measurement of externalised APL was conducted using the LC/MS/MS method described in Chapter 2 on separate occasions for UBD and DVT patients. The LC/MS/MS analysis of biotinylated lipid extracts from 16 healthy controls and 20 UBD patients was completed in July 2018. The remaining analyses of 10 healthy controls and the 22 thrombosis patients was conducted approximately 12 months later. It was anticipated that biotinylated standards would remain stable over the course of the study period allowing the data to be combined over the two runs. The original method stipulates that the standard curve can be reused for up to 3 months if stored at -80 ^{0}C and that standards can be stored for up to 1 year [153]. Beyond this however, experience of the stability of the biotinylation assay over a longer time period is unknown. In this study, the synthesised biotinylated standards were stored over a period of 2.5 years during recruitment, and separate standard curves were synthesised from main stocks for every LC/MS/MS analysis as recommended.

Two examples of a biotinylated PE and PS species from each run are shown in Figure 5.2. It was observed that the biotinylated standards from the initial stock used to generate the standard curves, resulted in significantly lower gradients for each analyte in the second run (C-D) performed in July 2019 compared with those performed in July 2018 (A-B). The gradients and R^2 values for each biotinylated lipid are shown in Table 5.1.

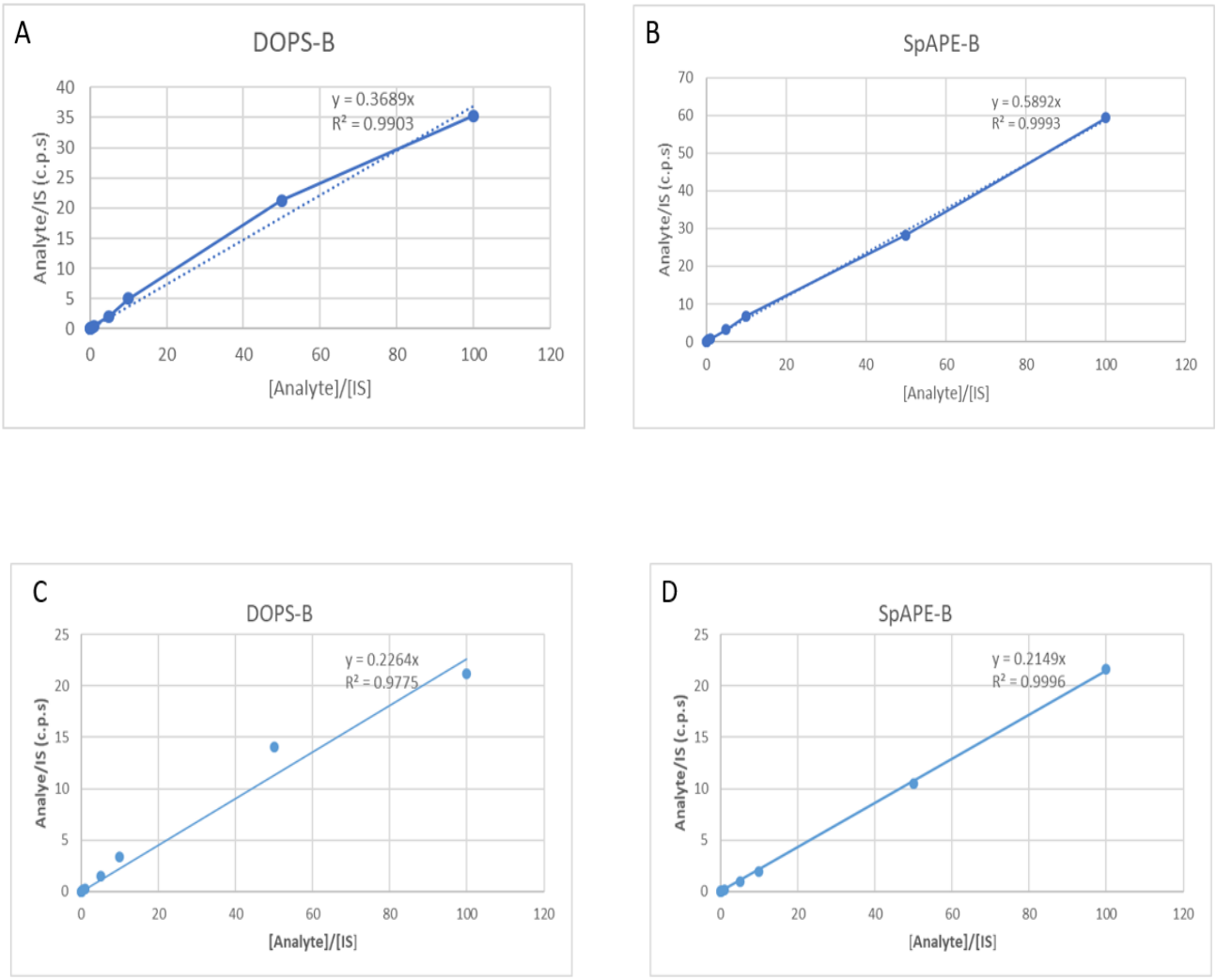
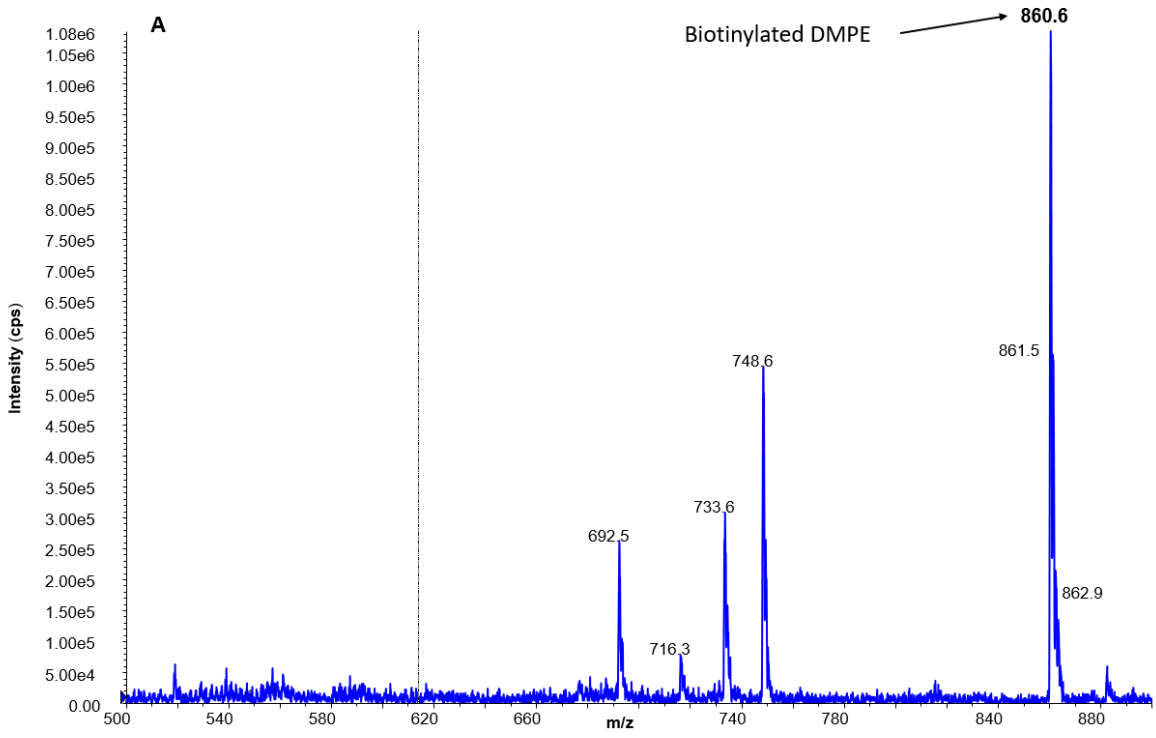


Figure 5.2 Standard curves for biotinylated species of PS and PE from two separate analyses 12 months apart. Run 1 (A-B) demonstrates the standard curve that was generated from biotinylated lipid stocks to quantify samples analysed in July 2018. The corresponding standard curves for the same species from Run 2 (C-D) were generated from the same lipid stocks approximately 12 months later and have noticeably lower gradients. The gradient (y) and R^2 value for each standard curve is shown.

Table 5.1 Gradients of standard curves with time. Run 1 and Run 2 represent LC/MS/MS analysis of the standard curves that were run with samples over the study period. These independent runs were conducted approximately 12 months apart.

Biotinylated species	Gradient of standard curves in Run 1 (July 2018)	Gradient of standard curves in Run 2 (July 2019)
DOPS-B	y=0.3689x R^2=0.9903	y=0.2264 R^2= 0.9775
SOPS-B	y=0.5233x R^2=0.9921	y=0.1940 R^2=0.9662
SAPS-B	y=0.4906x R^2=0.9907	y=0.1594 R^2=0.9746
SOPE-B	y=0.6383x R^2=0.9986	y=0.1347 R^2=0.9998
SpAPE-B	y=0.5892x R^2=0.9993	y=0.2149 R^2=0.9996
SAPE-B	y=0.5319x R^2=0.9971	y=0.1179 R^2=0.9998

Analysis of PS and PE biotinylated standard by using direct infusion and Q1 scans were performed which confirmed the characteristic fragmentation pattern of the biotinylated lipid with no native species detected, excluding the possibility of degradation or loss of biotin (Figure 5.3).



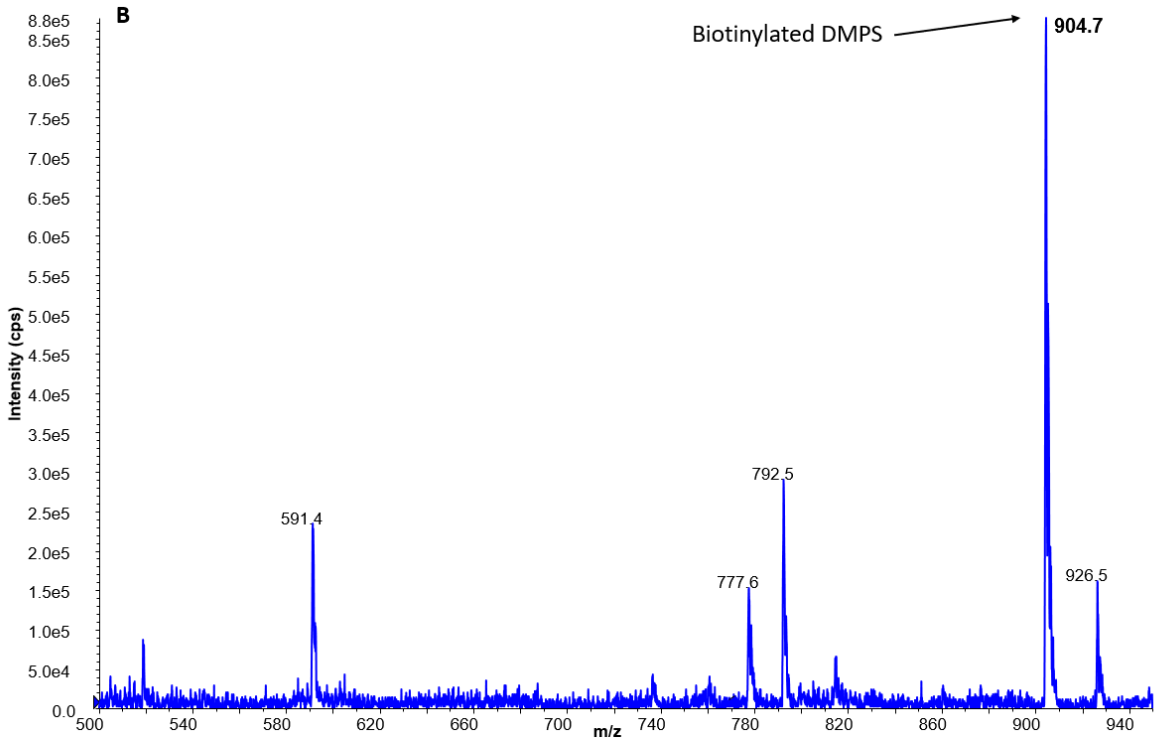


Figure 5.3 Q1 scans for biotinylated PE and PS species (A) Biotinylated DMPE (*m/z* 860 $[M-H]^-$) is seen on direct infusion with no evidence of the native PE species (*m/z* 634 $[M-H]^-$). (B) Biotinylated DMPS (*m/z* 904 $[M-H]^-$) is seen on direct infusion with no evidence of the native PS species (*m/z* 678 $[M-H]^-$).

The two primary and six internal standards from initial stocks were subsequently re-weighed following their synthesis. Biotinylated standards had been synthesised to a concentration of 1 mg/ml and were re-weighed approximately 2.5 years later, at the end of the study recruitment period to investigate the change in gradients of the standard curves. For each biotinylated lipid, a known amount was transferred to a pre-weighed glass vial and then dried with liquid nitrogen. The glass vial was then re-weighed and the difference in weights recorded. All biotinylated standards had lower weights than those recorded initially following their synthesis. It is possible that due to their inherent stickiness as lipids, they may have stuck to the glass vial thus accounting for their change in concentration over time.

Table 5.2 Re-weighed primary and internal biotinylated standards over a timescale of 2.5 years

Original stock 1 mg/ml	Pre-weighed vial	Amount of lipid transferred to vial	Weight of vial with dried lipid	Amount of lipid after re-weighing	% of original weight
DMPE-B	2613.605 mg	600 µg	**2614.043 mg**	**438 µg**	**73 %**
SOPE-B	2598.303 mg	600 µg	**2598.762 mg**	**459 µg**	**76.5 %**
SpAPE-B	2606.808 mg	500 µg	**2607.206 mg**	**398 µg**	**79.6 %**
SAPE-B	2618.849 mg	500 µg	**2619.225 mg**	**376 µg**	**75.2 %**
DMPS-B	2591.656 mg	600 µg	**2592.121 mg**	**465 µg**	**77.5 %**
DOPS-B	2613.458 mg	700 µg	**2613.847 mg**	**389 µg**	**55.6 %**
SOPS-B	2617.350 mg	500 µg	**2617.726 mg**	**376 µg**	**75.2 %**
SAPS-B	2636.851mg	400 µg	**2637.154 mg**	**303 µg**	**74.5 %**

5.3 Results

5.3.1. Measurement of native APL in healthy controls obtained from independent LC/MS/MS analyses

As discussed in Chapter 2, methods for quantification of lipids require the standard curve to be run simultaneously and analysed with the samples of interest, however for Run 2 this would have yielded discrepant results due to the change in concentrations of the standards with time. The standard curve generated in July 2018 was therefore used for the quantification of externalised lipids in samples of Run 2. For each biotinylated lipid species apart from SpAPE, there was no difference in the amount of lipid externalised in the platelets of healthy controls from Run 1 (n=16) and Run 2 (n=10) using the original standard curves for quantification. Despite, the apparent normalisation of results shown using the original standard curve, the results from the separate runs have been analysed independently for the remainder of the chapter.

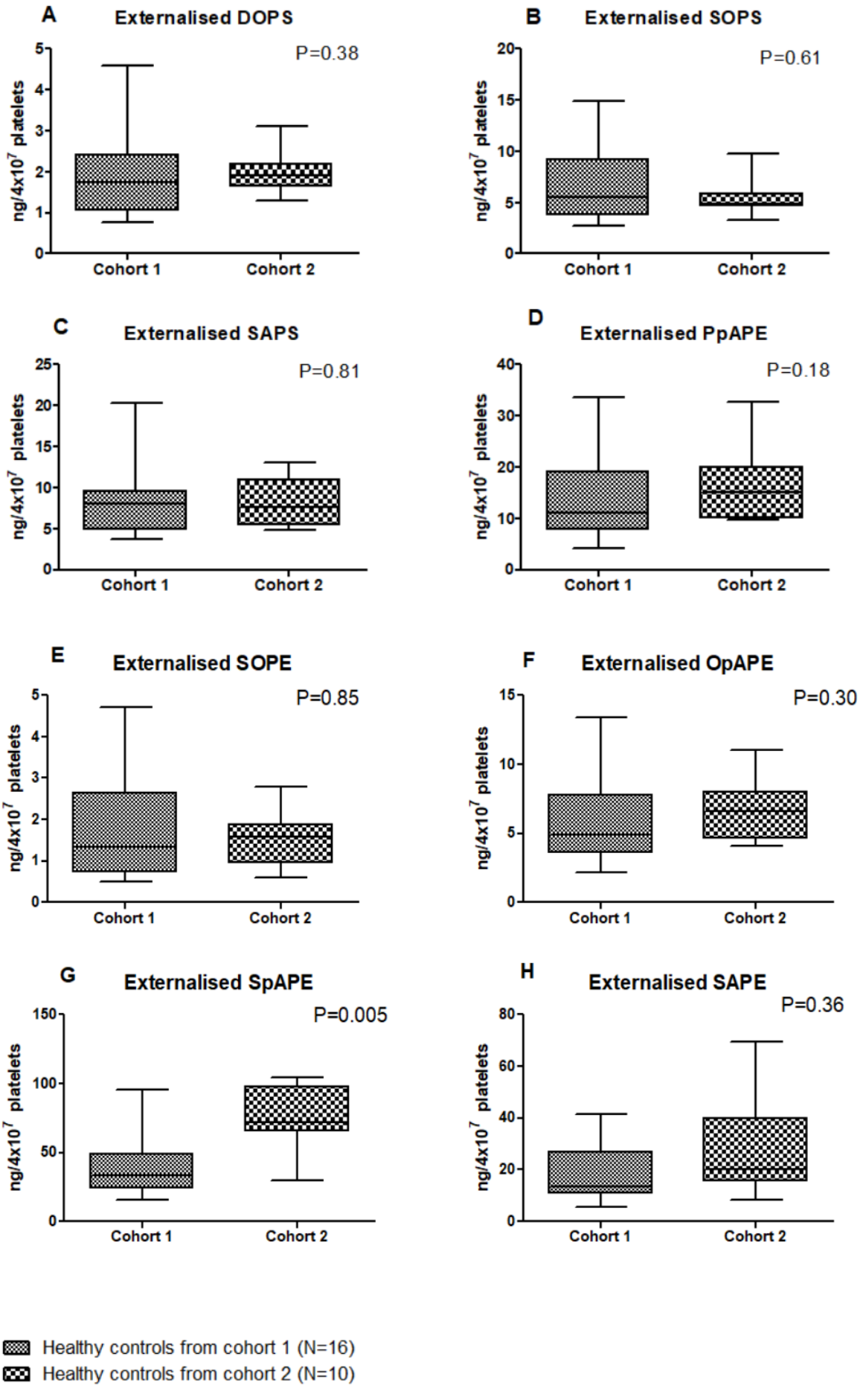

A
Externalised DOPS
P=0.38
ng/4x10^7 platelets
Cohort 1
Cohort 2
B
Externalised SOPS
P=0.61
C
Externalised SAPS
P=0.81
D
Externalised PpAPE
P=0.18
E
Externalised SOPE
P=0.85
F
Externalised OpAPE
P=0.30
G
Externalised SpAPE
P=0.005
H
Externalised SAPE
P=0.36
Healthy controls from cohort 1 (N=16)
Healthy controls from cohort 2 (N=10)

Figure 5.4 The quantity of externalised PE/PS species in the platelet membrane of healthy controls from Cohort 1 and Cohort 2 is the same using the original standard curve for quantification. For each biotinylated lipid species, the quantity of PE and PS externalised is shown using the standard curve generated in July 2018. The original standard curve was used in the LC/MS/MS analyses of Cohort 1 and was run with biotinylated lipid samples simultaneously. The standard curve used in Run 1 was used to retrospectively quantify the 10 healthy controls from Run 2 (Cohort 2) 12 months later due to the change in concentration of the biotinylated standards with time. P values were calculated using the Mann Whitney U test.

5.3.2 Characterisation of native APL and total platelet lipids in UBD patients.

Following thrombin activation of platelets, there was no significant difference in the amount of externalised PE/PS in healthy controls compared with UBD patients (Figure 5.5). This was also the case for basal concentrations of APL, which were measured in lower quantities compared with activated samples (Appendix 1). The total amount of PE and PS in the platelet membrane was the same irrespective of whether the platelet was in the resting state or following the addition of thrombin (Figure 5.6). It is notable that whilst the percentage of lipid externalised by the platelets of the Scott patient was lower than the average of both the UBD and healthy controls, it was comparable with some of the subjects in both cohorts suggesting that a minimal degree of APL exposure occurs on the surface of Scott syndrome platelets.

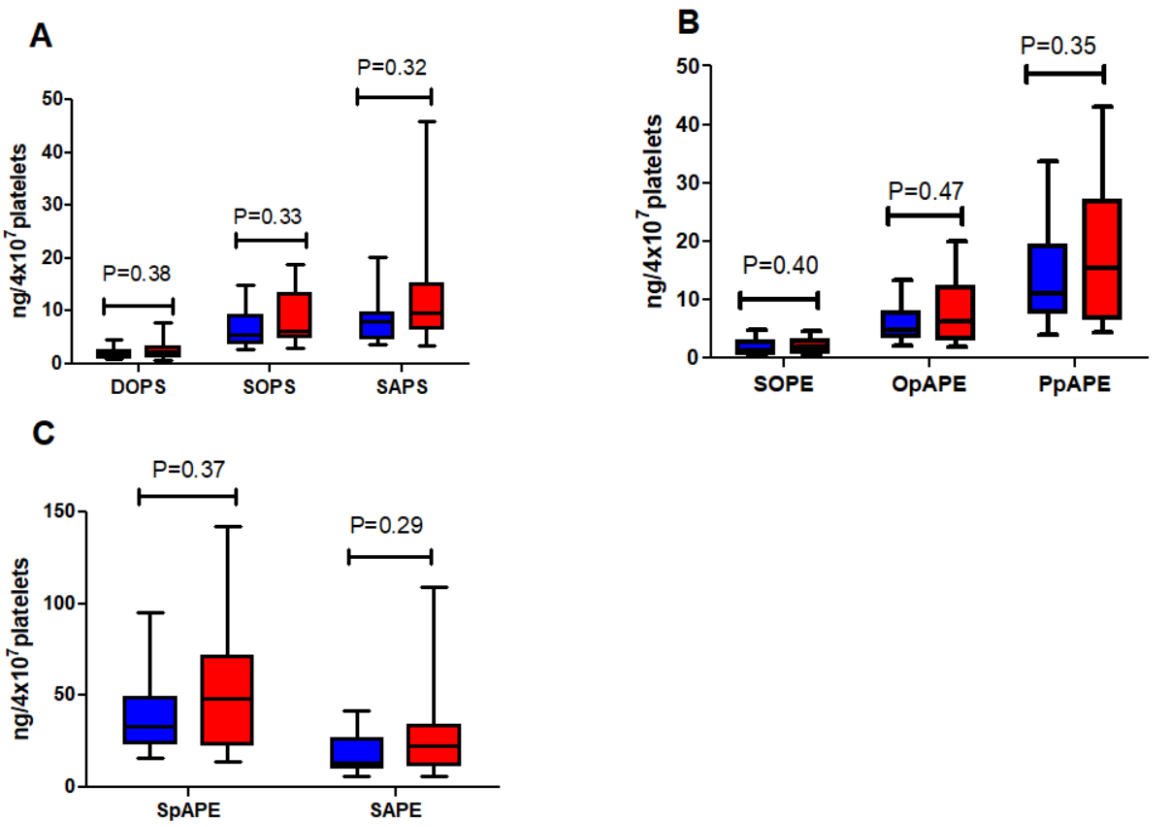


Figure 5.5. Externalised PE and PS species following thrombin activation in platelets of healthy controls and UBD patients is similar. Washed platelets were isolated and activated with thrombin. External facing APL were biotinylated using the cell impermeable reagent SNB and then extracted using a Bligh and Dyer method as described in Section 2.11. Lipid extracts were then analysed using LC/MS/MS and quantitated as detailed in Chapter 2. Individual species of externalised PS are shown for healthy controls (blue) and patients with UBD (red) (A). The quantities of the most abundant species of PE (B) and the three least abundant species of PE (C) are shown. Data shown are median values (horizontal black bar), interquartile range (box) and maximum and minimum values (whiskers). P values were calculated using the Mann Whitney U test.

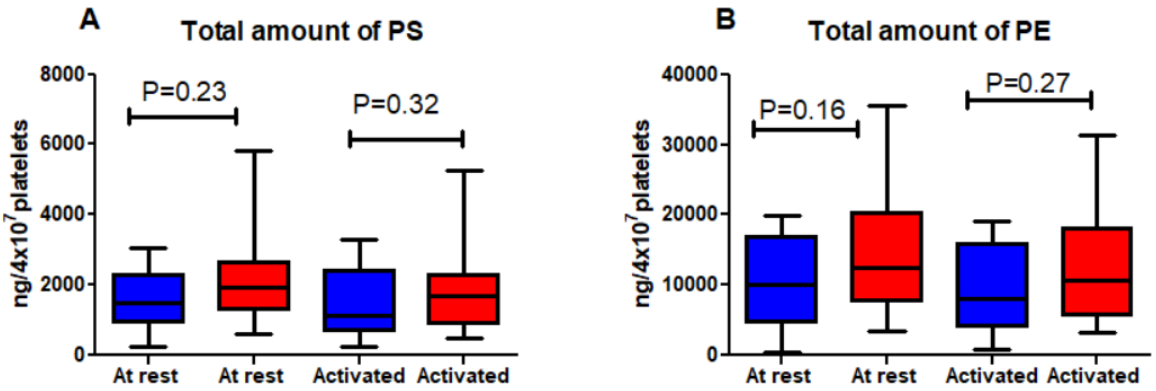


Figure 5.6 The total amount of PS and PE measured in the platelet membrane basally or following thrombin activation is the same in UBD patients and healthy controls. The cell permeable reagent, NHS-Biotin was added to an individuals' washed platelets in the unactivated state and following activation with thrombin. Biotinylated total cellular lipids were then extracted and analysed using LC/MS/MS as detailed in Section 2.13, Chapter 2. Individual molecular species of PE and PS were quantitated and then combined to determine the total quantity of PE and PS. The total amount of PS and PE measured in the resting and activated platelet membrane for both healthy controls (blue) and UBD patients (red) is shown (A-B). Data shown are median values (horizontal black bar), interquartile range (box) and maximum and minimum values (whiskers). P values were calculated using the Mann Whitney U test.

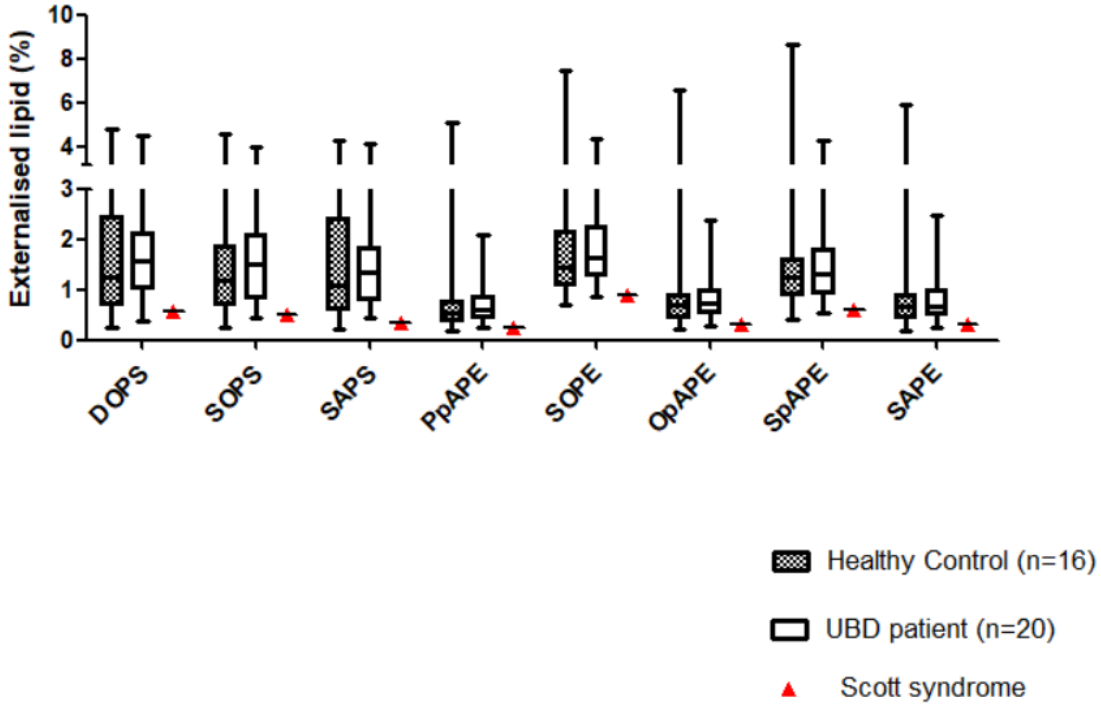
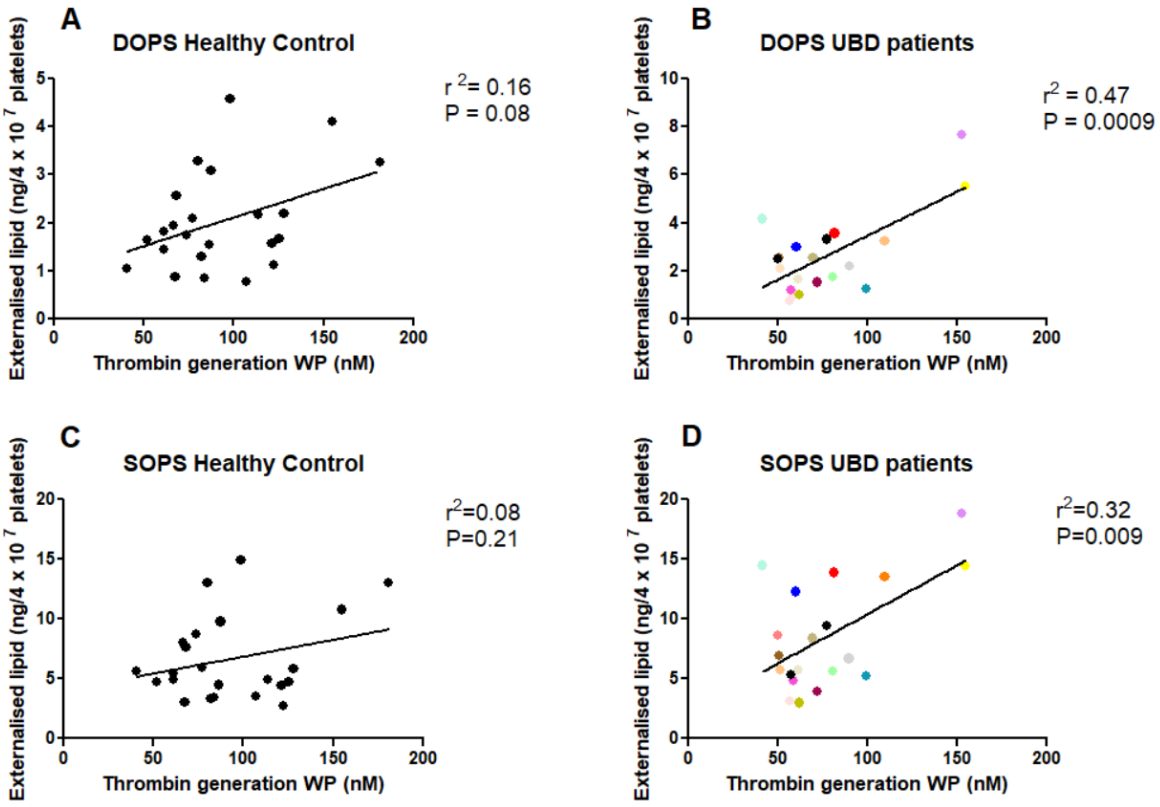
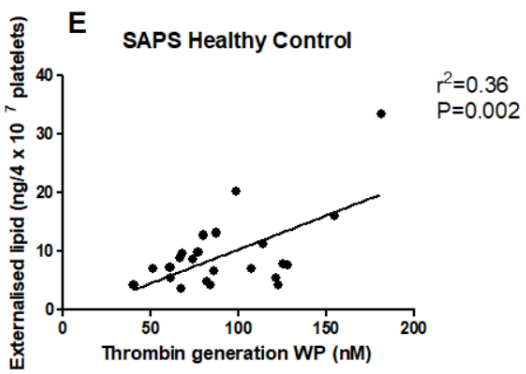


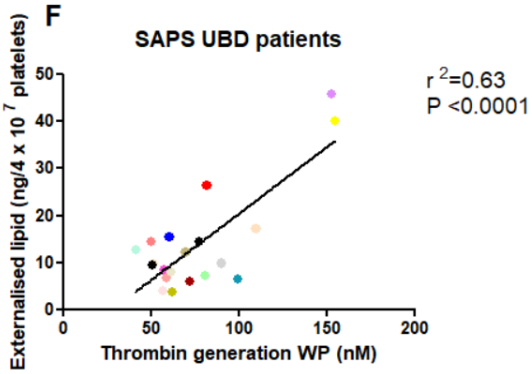
Figure 5.7 The percentage (%) of externalised PE and PS following thrombin activation in platelets of healthy controls and UBD patients is the same. The biotinylation of external APL using SNB and total lipids using cell permeable NHS-biotin enables the percentage of each individual species of PE and PS to be determined. Data from the Scott patient is depicted by the red triangle. Data shown are median values (horizontal black bar), interquartile range (box) and maximum and minimum values (whiskers). There were no statistically significant differences between healthy controls and UBD patients.

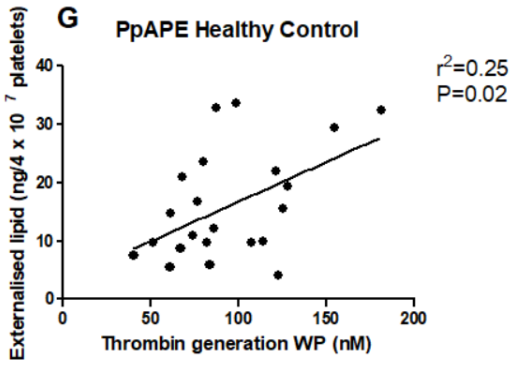
5.3.3 Correlation of peak thrombin generation with externalised PE and PS in UBD patients.

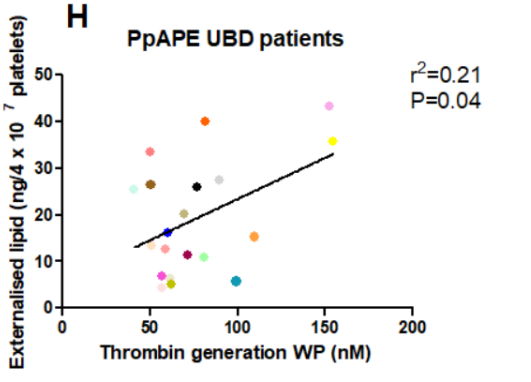
Subsequently, a correlation between peak thrombin generation from washed platelets (Chapter 4) with externalised PE and PS was investigated. A weakly positive correlation was observed in healthy controls and a moderate correlation was seen in the UBD cohort. The strongest correlations were evident in the UBD cohort and were more marked for the abundant lipid species (SAPS, SpAPE and SAPE) (F, N and P).

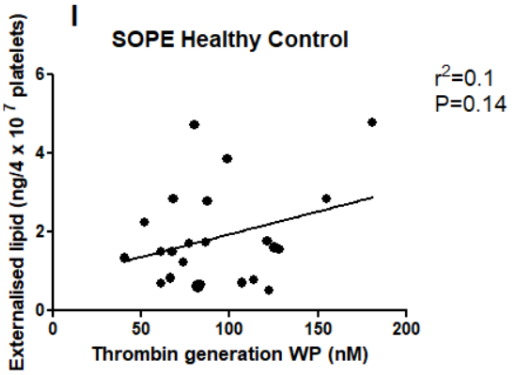


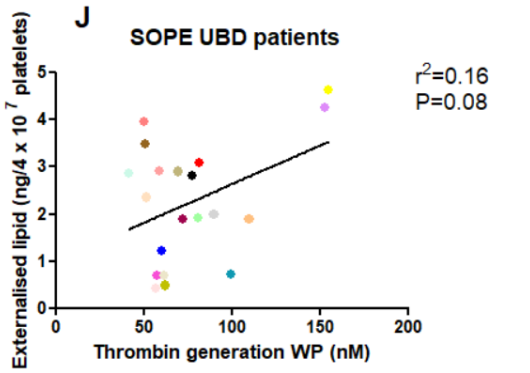

E
SAPS Healthy Control
r2=0.36
P=0.002
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)

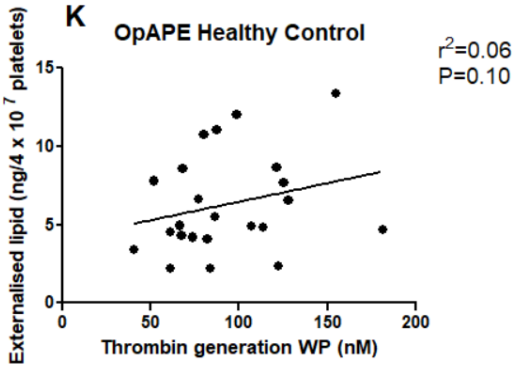

F
SAPS UBD patients
r 2=0.63
P <0.0001
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)

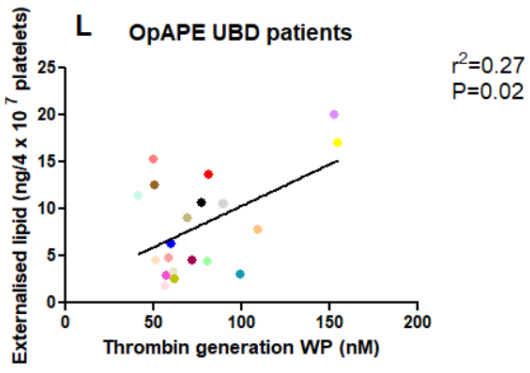

G
PpAPE Healthy Control
r2=0.25
P=0.02
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)


H
PpAPE UBD patients
r2=0.21
P=0.04
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)


I
SOPE Healthy Control
r2=0.1
P=0.14
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)


J
SOPE UBD patients
r2=0.16
P=0.08
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)


K
OpAPE Healthy Control
r2=0.06
P=0.10
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)


L
OpAPE UBD patients
r2=0.27
P=0.02
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)

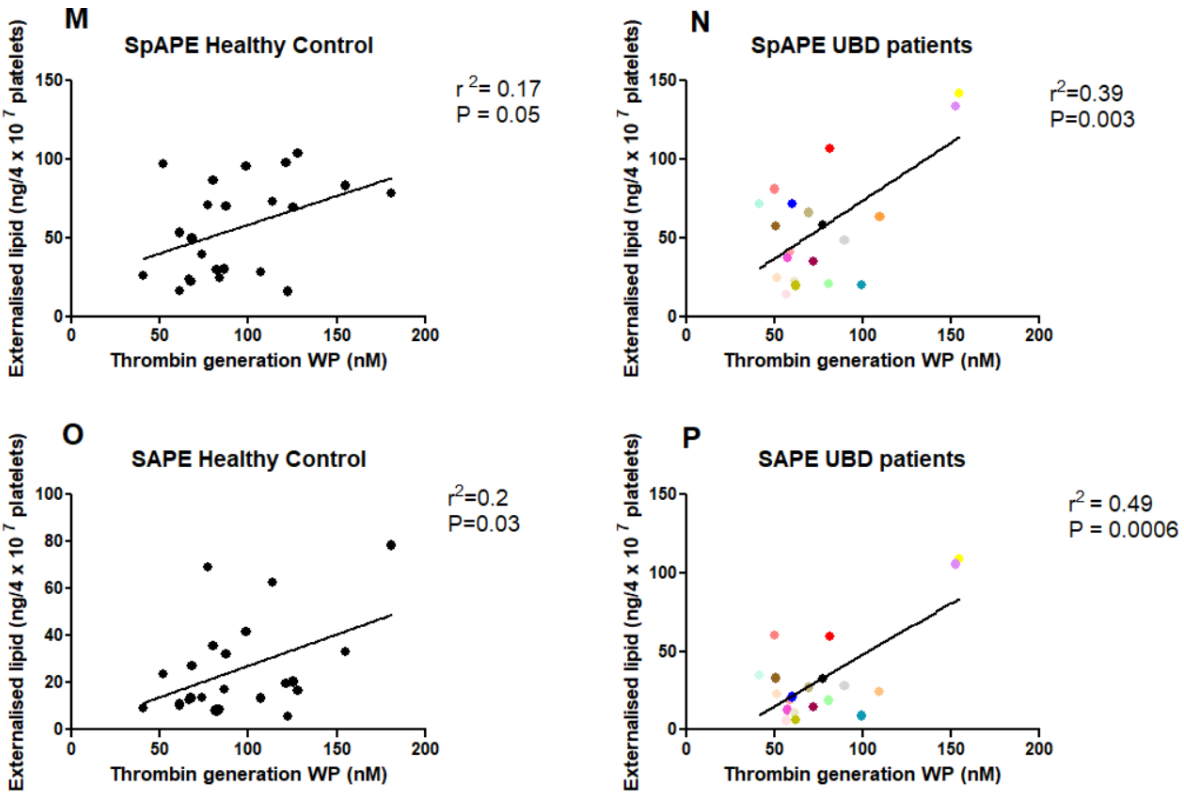
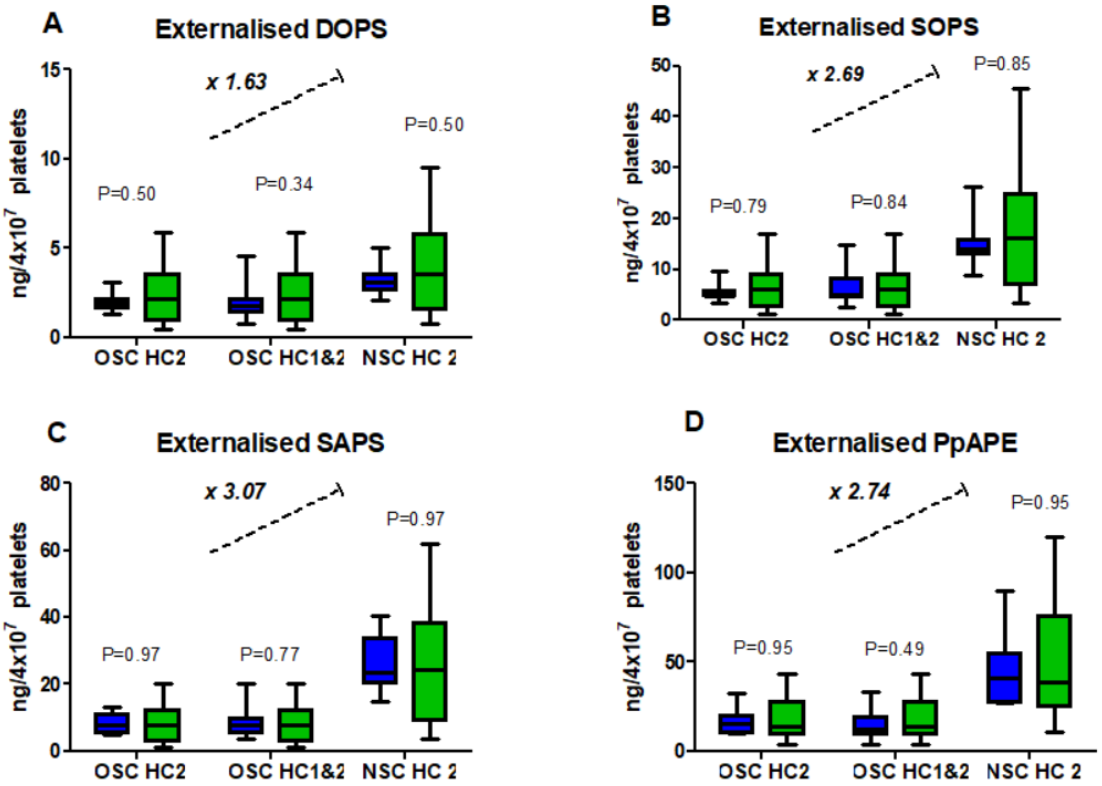


Figure 5.8 Positive correlations of externalised PE/PS species and peak thrombin generation supported by washed platelets are shown in healthy controls and UBD patients. The linear correlation for each species of PE and PS with peak thrombin generation is shown from the most abundant lipid to the least for both healthy controls (black dots) and the UBD cohort (coloured dots). The peak thrombin measurements were derived using the individuals' washed platelets (1.5 x 10^8/ml) and a standardised liposome (0.5 µM phospholipid/10 pM TF) using the thrombin generation assay described in Section 4.2.1, Chapter 4. For each correlation plot, the correlation co-efficient (r squared) with the corresponding p-value (P) is shown. The Scott syndrome patient has not been included in the analysis.

5.3.4 Characterisation of native and total platelet lipids in DVT patients

The quantity of externalised PE and PS in thrombin-activated platelets was similar between healthy controls and patients with DVT (Figure 5.9). This was observed for each individual species of PS and PE when DVT patients were compared with healthy controls from the same run (i.e. Run 2) and in combination with healthy controls from Run 1. The original standard curve was used for the quantification of externalised lipid due to the decrease in standards with time, however the corresponding data generated from standard curves run with the samples (i.e. Run 2) is also shown for comparison. The fold change between results using the gradients of the two sets of standard curves (Table 5.2) is shown for each biotinylated lipid. Similarly, basal amounts of externalised APL in the platelet membrane of DVT patients were not increased compared with healthy controls suggesting that higher quantities of PE/PS were not contributory to the thrombotic phenotype of these patients (Appendix 1).



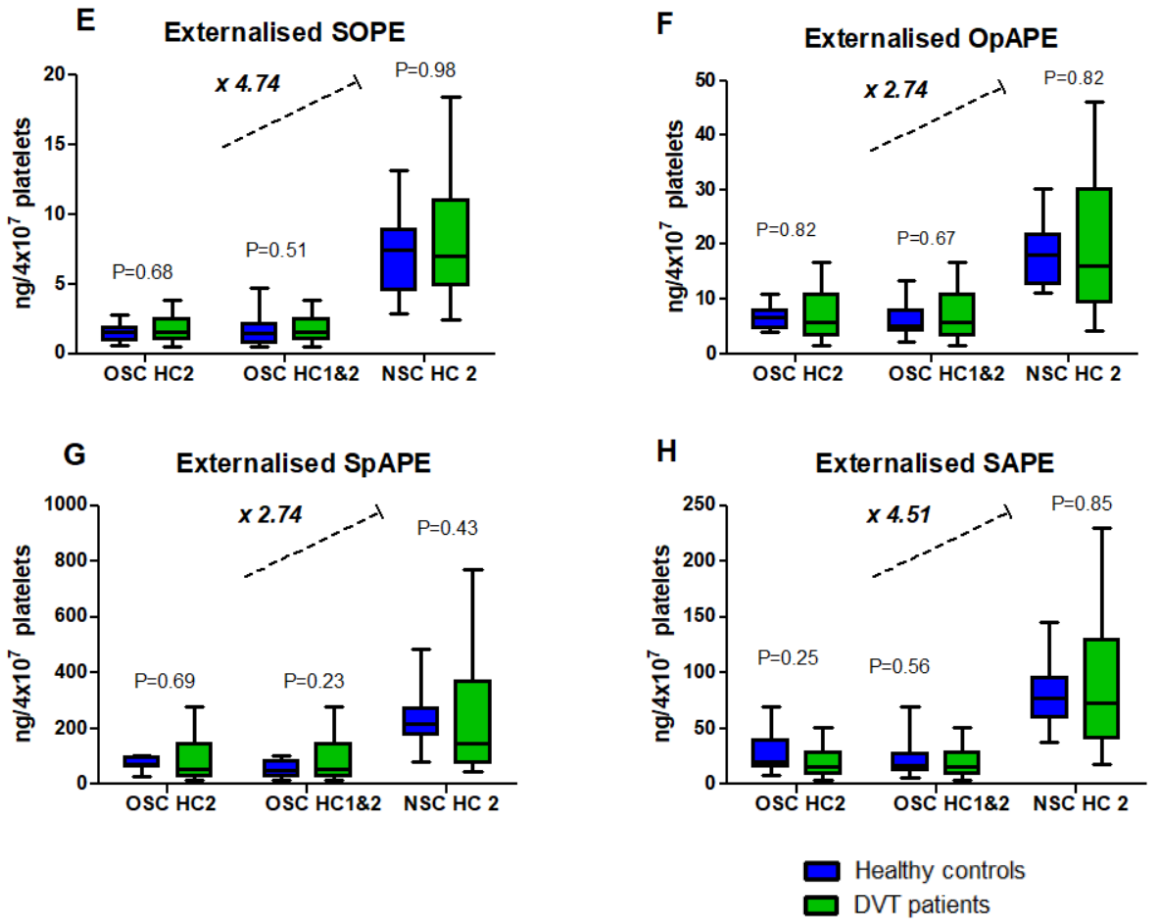


Figure 5.9 Externalised PE and PS species following thrombin activation in platelets of healthy controls and DVT patients is similar. Individual species of externalised PS and PE are shown for healthy controls (blue) and patients with DVT (green) (A-H). The quantities of individual species of PE and PS have been quantitated using the original standard curve (OSC) from Run 1 and also the new standard curve (NSC) from Run 2. The factor by which the results differ using the two sets of standard curve is shown for each biotinylated lipid. Data shown are median values (horizontal black bar), interquartile range (box) and maximum and minimum values (whiskers). P values were calculated using the Mann Whitney U test.

The total amount of PS and PE in the platelet membrane was the same in the resting state and following thrombin activation (Figure 5.10). No difference was observed in the percentage of externalised lipid between healthy controls and DVT patients for all biotinylated species except for SAPE (Figure 5.11). Although DVT patients externalised similar amounts of SAPE as shown in Figure 5.9, the percentage of externalised SAPE appears to be significantly lower due to a relatively high abundance of this lipid in the platelet membrane in thrombotic patients. In one outlying DVT patient, there was a higher percentage of biotinylated lipid externalised, however this is because the total quantity of PS and PE extracted from the platelets of this patient was abnormally low and is likely to represent an incomplete lipid extraction.

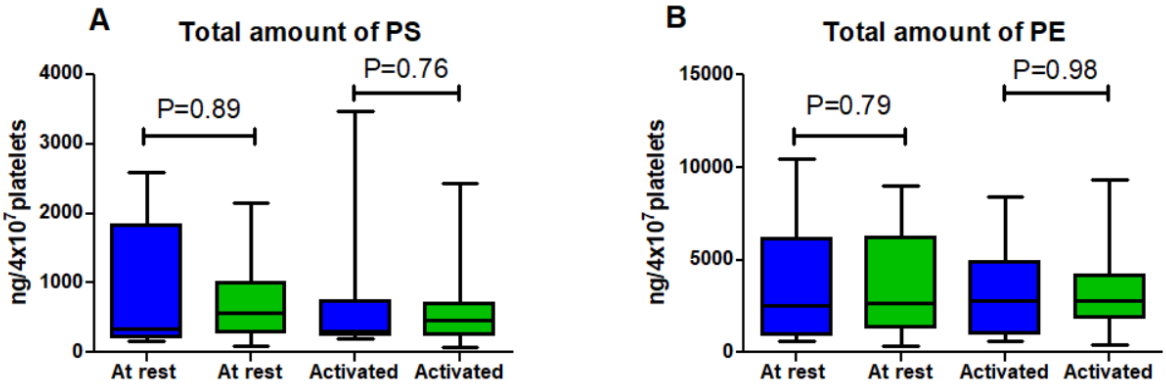


Figure 5.10. The total amount of PS and PE measured in the platelet membrane (at rest or following thrombin activation) is the same in DVT patients and healthy controls. The total quantity of PS and PE measured in the resting and activated platelet membrane for both healthy controls (blue) and UBD patients (green) is shown (A-B). Data shown are median values (horizontal black bar), interquartile range (box) and maximum and minimum values (whiskers). P values were calculated using the Mann Whitney U test.

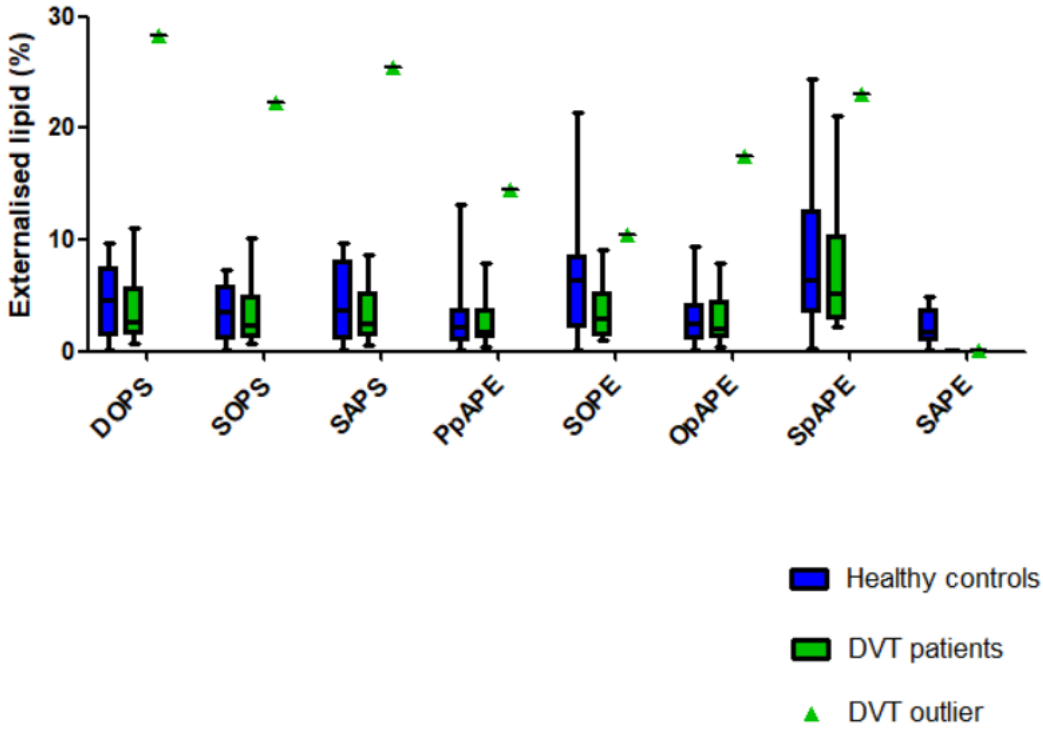
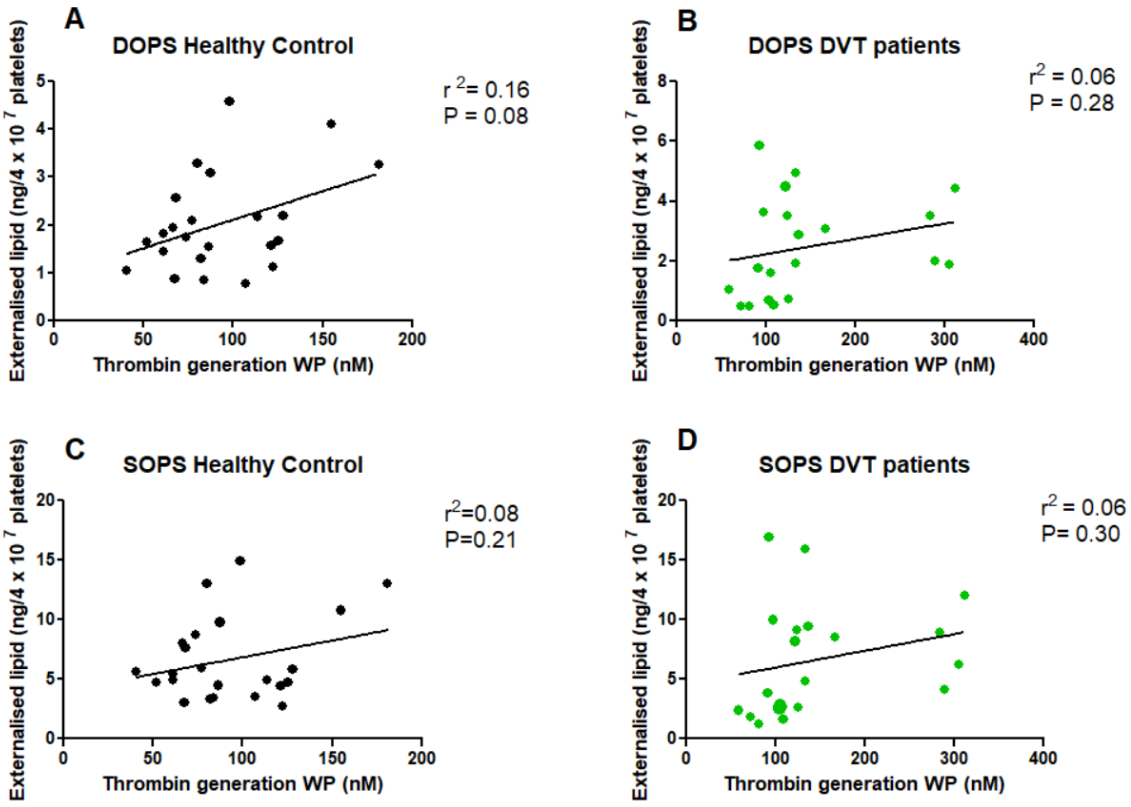
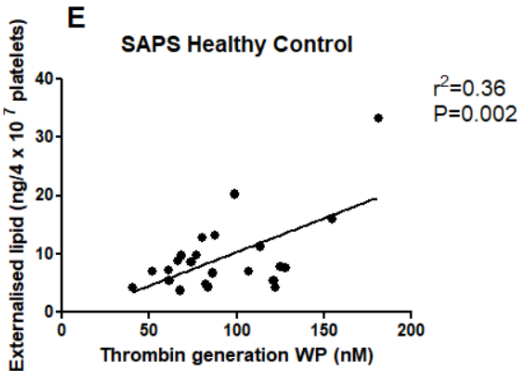


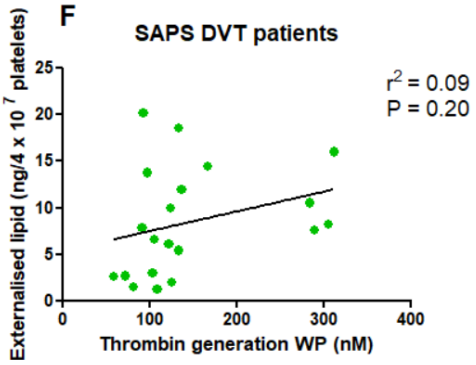
Figure 5.11 The percentage (%) of externalised PE and PS following thrombin activation in platelets of healthy controls and DVT patients is the same. The percentage of each individual species of PE and PS is shown. Data from the an outlying DVT patient is depicted by the green triangle. Data shown are median values (horizontal black bar), interquartile range (box) and maximum and minimum values (whiskers). There were no statistically significant differences between healthy controls and DVT patients.

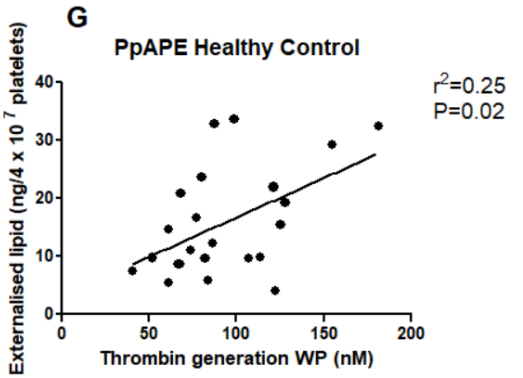
5.3.5 Correlation of peak thrombin generation with externalised PE and PS in DVT patients.

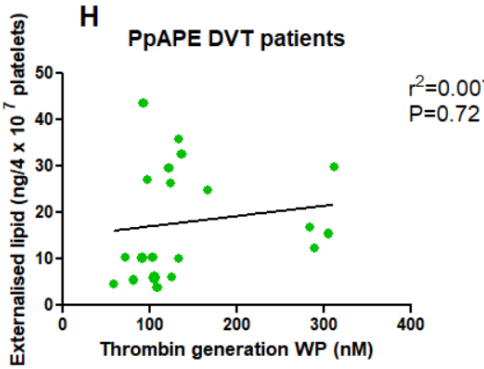
Whilst the peak thrombin generated from washed platelets in the control group showed a weak positive correlation with externalised PE and PS, no correlation was observed in the thrombotic cohort (Figure 5.12). Furthermore, the values in each scatter plot were not uniformly distributed with 4 thrombotic outliers identified in the thrombin generation assay, forming a separate and distinct cluster from others within the DVT cohort. Despite generating significantly higher amounts of peak thrombin in the thrombin generation assay, these 4 patients were shown to externalise only modest quantities of PE and PS. This shows that platelet associated factors other than externalised lipids are important in promoting the enhanced support for global coagulation seen in these patients and potentially contributing to the thrombotic episodes in these patients.

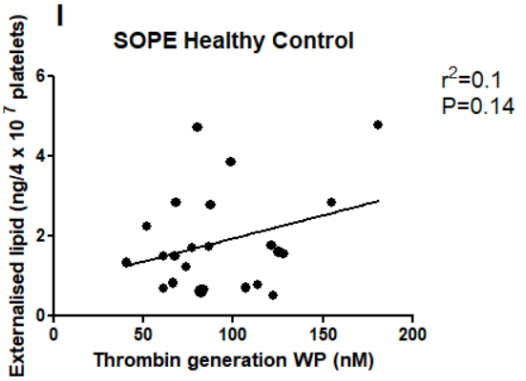


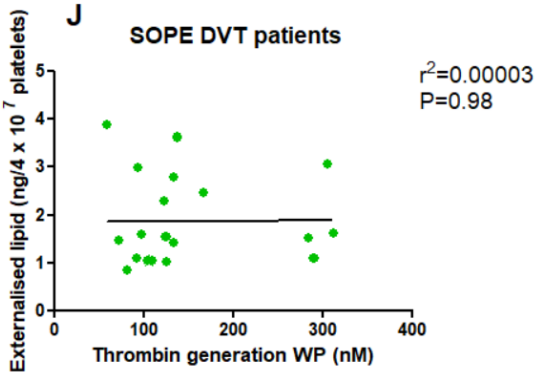

E
SAPS Healthy Control
r2=0.36
P=0.002
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)

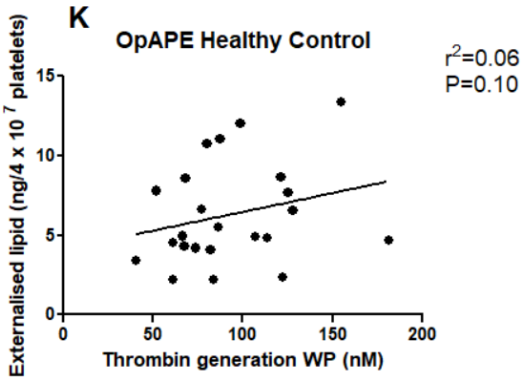

F
SAPS DVT patients
r2 = 0.09
P = 0.20
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)

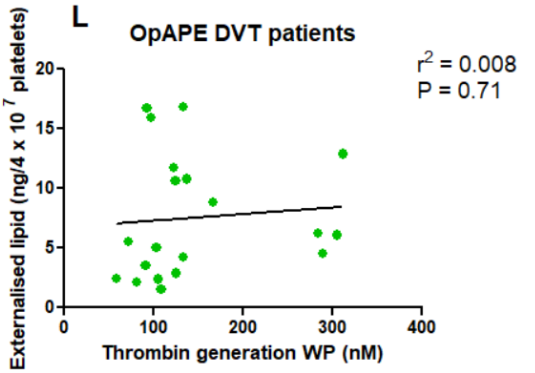

G
PpAPE Healthy Control
r2=0.25
P=0.02
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)


H
PpAPE DVT patients
r2=0.007
P=0.72
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)


I
SOPE Healthy Control
r2=0.1
P=0.14
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)


J
SOPE DVT patients
r2=0.00003
P=0.98
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)


K
OpAPE Healthy Control
r2=0.06
P=0.10
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)


L
OpAPE DVT patients
r2 = 0.008
P = 0.71
Externalised lipid (ng/4 x 10 7 platelets)
Thrombin generation WP (nM)

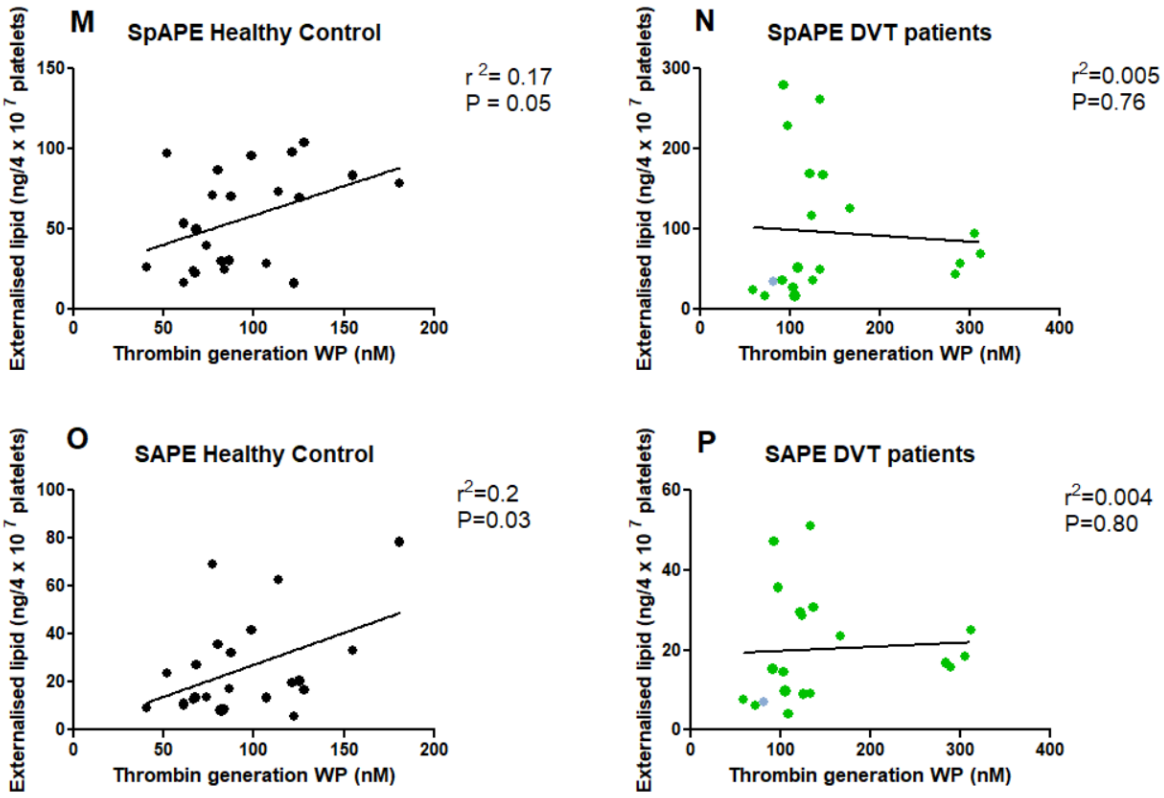


Figure 5.12 Externalisation of PE/PS in thrombin-activated platelets does not correlate with peak thrombin generation supported by washed platelets of DVT patients. The linear correlation for each species of PE and PS with peak thrombin generation is shown from the most abundant lipid to the least for both healthy controls (black dots) and DVT patients (green dots). The peak thrombin measurements were derived using the individuals' washed platelets (1.5 x 10^8/ml) and a standardised liposome using the thrombin generation assay described in Section 4.2.2, Chapter 4. For each scatter plot, the correlation co-efficient (r squared) is shown with the corresponding p-value (P).

5.4 Discussion

This aim of this chapter was to examine whether differences in PE/PS externalisation in thrombin-activated platelets of patients with UBDs and venous thrombosis differ from healthy controls and are potentially contributory to the pathogenesis of these disorders. The importance of APL exposure in supporting coagulation in human disease has previously been demonstrated in Scott syndrome, whereby failure of platelets to externalise PE/PS due to defective scramblase activity results in bleeding symptoms [95]. It was therefore hypothesised that the platelets of UBD patients may have reduced ability to externalise PE and PS akin to a mild form of Scott syndrome. However, the quantities of PE or PS externalised following thrombin activation were similar between the UBD patients and healthy controls providing no support for this hypothesis. Similarly, no changes in PE or PS externalisation were observed in thrombotic patients compared with healthy controls.

It is well established that the exposure of PS on the activated platelet surface membrane is essential for the interaction with calcium and the Gla domains of Vitamin K dependent clotting factors thus promoting coagulation in vitro. The function of PS is enhanced by PE [106, 107]. This is supported by the data from the Scott syndrome patient reported here where very low levels of thrombin generation are associated with limited externalisation of PS and PE. The data presented in this chapter show that externalisation of PS and PE does not contribute to the excessive bleeding in UBDs or to increased coagulation in patients with venous thrombosis. This means that the reduced ability of washed platelets to support peak thrombin generation reported in Chapter 4 in UBD patients cannot be explained by decreased externalisation of PS or PE and other mechanisms need to be sought.

Apart from Scott syndrome the APL composition of the platelet membrane has not been extensively investigated in other bleeding or thrombotic disorders to date. Recent work has demonstrated increased numbers of PS- positive platelets in hypercoagulable conditions including cancer and essential thrombocythaemia although to date this has been limited to techniques such as annexin V or lactadherin binding. These techniques confirm the presence of externalised PS or PE rather than measuring the actual quantity of individual species as conducted in this study for the first time [204, 237]. There is increasing evidence to suggest that other blood cells and their interaction with platelets are important in thrombus formation. For example, eosinophils were recently shown to expose APL on ADP activation, thus enhancing thrombin generation [238]. Furthermore, it was reported that the contact of platelets with red blood corpuscles is important in mediating PS exposure on red blood cells and promoting platelet activation and adhesion in pathological thrombi through FAS-L/FAS-R interactions [239]. As demonstrated here, increased APL exposure was not observed in platelets of DVT patients when they were activated and investigated following their isolation from whole blood.

The second aim of this chapter was to investigate the contribution of externalised species of PE and PS to the measured peak thrombin generation. Moderately positive correlations were demonstrated in the UBD cohort showing that the thrombin generation assay performed on washed platelets was, at least in part, sensitive to the amount of externalised PS and PE. However, when the amount of externalised PS and PE was directly compared it was found to be similar in the UBD patients and the healthy controls (Figure 5.5 and 5.7). This suggests that the decreased ability of washed platelets from patients with UBDs to support peak thrombin generation was not due to decreased externalisation of PS and PE.

Positive correlations between peak thrombin and native phospholipids were also seen in healthy controls, although the associations tended to be weaker than for the bleeding disorder cohort. Possible explanations for weaker correlations in the controls could include the age and gender difference between the disease and control group. It is notable that there was a clear female predominance in the bleeding cohort (18 females, 2 males), most of whom were within a similar age group (45-81 years). This contrasts with the control group, which consisted of a more equal gender mix (16 females, 13 males) spanning over a wider age range (21- 69 years). The effect of age and gender on the externalisation of platelet lipids is unknown although reports of an association with gender exist in the generation of oxidised phospholipids in human platelets[240].

No association was found between the amount of externalised APL and peak thrombin generation in the thrombotic group. This suggested that the thrombin generation assay was not sensitive to externalised PS/PE in this situation and that the increased ability of the platelets of thrombosis patients to support global coagulation was not primarily due to

increased externalisation of phospholipids. This was confirmed when the amounts of externalised PS/PE were directly measured (Figure 5.8 and 5.10) as similar amounts were found in the thrombosis patients and healthy controls. The increased ability of the platelets from the thrombosis patients to support thrombin generation was likely, therefore, to be caused by mechanisms other than externalised PS/PE and further studies will be required to investigate this.

The four thrombosis patients whose platelets supported substantially higher amounts of thrombin generation compared to other patients in the cohort and controls had widely variable amounts of externalised phospholipids. It is important to note that samples for thrombin generation and lipid analysis were collected separately on consecutive days and as discussed previously, a higher incidence of spontaneous platelet aggregation occurred in DVT patients suggesting a degree of platelet hyper-reactivity. This implies that platelets in patients at the time of thrombosis may behave in a less predictable or uniform manner compared to non-thrombotic states. Differences in the in-vivo environment may account for this for example exposure to local pro-inflammatory stimuli which are considered to be influential in the formation of venous thromboembolism [241].

In contrast to platelets, there is extensive evidence supporting the role of platelet and other cell-derived microvesicles in promoting both arterial and venous thrombosis. Experiments determining the quantities of individual PE and PS species from platelet-derived microvesicles of patients within the thrombotic or bleeding cohorts were not carried out here, and further work is needed to investigate this fully. The experiments conducted in this chapter did not identify changes in APL externalisation in thrombin-activated platelets that could account for the bleeding or thrombotic symptoms of the two independent cohorts. It is therefore possible that changes in other lipids in the platelet membrane may be influential in UBD and thrombotic patients. Further research is required to evaluate these findings in other bleeding and arterial thrombotic disorders, as well as examine the inter-relationship between APL and oxidised lipids in haemostatic disorders.

In conclusion the changes in thrombin generation described in patients with UBDs and venous thrombosis cannot be attributed to externalisation of unoxidised PS and PE and other mechanisms needed to be considered. Therefore, the potential role of eoxPLs was investigated and this is described in the next chapter.

Chapter 6 - The generation of eoxPL by activated human platelets in UBD and DVT patients

6.1 Introduction

It has been almost a decade since a family of oxidized phospholipids were shown to be generated enzymatically via 12-LOX in human platelets. Specifically, thrombin-activated platelets were demonstrated to generate six molecular species of 12-HETE-PLs, consisting of four 12-HETE-PEs ((PE (16:0p_12-HETE), PE(18:1p_12-HETE), PE(18:0p_12-HETE) and PE(18:0a_12-HETE-PE)) and two 12-HETE-PCs (PC(16:0a_12-HETE, PC(18:0a_12-HETE-PC)) as described in Chapter 1 [134]. Since this discovery, there have been further advances in the understanding of the role of eoxPL in haemostasis and thrombosis.

Following observations that both 12-HETE-PEs and 12-HETE-PCs could promote coagulation by enhancing the function of externalised PS in vitro, subsequent studies in vivo have supported these findings [146, 149]. Firstly, it was shown that mice lacking the *Alox15* or *Alox12* gene were more susceptible to haemorrhage after a haemostatic challenge and that injection of 12-HETE-PE and 12-HETE-PC resulted in a reduction in bleeding [146]. In human platelets from patients with antiphospholipid syndrome and venous thrombosis, increased amounts of 12-HETE-PE were measured in lipid extracts compared with those from healthy controls [146]. In patients post cardiopulmonary bypass (CPB) who have an increased risk of bleeding, 12-HETE-PE generation was shown to be reduced in platelets compared to pre-operatively [149]. Thus, there is increasing evidence to suggest that HETE-PLs have a role in maintaining haemostasis and that they may be implicated in the pathogenesis of bleeding and thrombotic disorders.

Methods of analysing HETE-PLs in previous studies have used a targeted lipidomics approach using LC/MS/MS on a Q-Trap instrument (Applied Biosystems 4000 Q-Trap) operating in the negative mode [134]. The same method of analysis was used for the work described in this chapter. Products were analysed using multiple reaction monitoring (MRM) enabling the detection of several compounds at the same time by monitoring specific transitions from the parent ion to the daughter ion (*m/z* 179.2).

The experiments in this chapter sought to identify whether there were differences in the amounts of HETE-PLs generated by platelets from UBD and DVT patients without an associated prothrombotic condition such as antiphospholipid syndrome.

6.2 Results

6.2.1 Identification of 12-HETE-PLs generated by thrombin-activated platelets

To characterise 12-HETE-PL generation, isolated platelets were left unstimulated or activated with thrombin and incubated for 30 minutes prior to lipid extraction in UBDs, DVT patients and in healthy controls. The four molecular species of 12-HETE-PE and the two molecular species of 12-HETE-PC were identified with precursor LC/MS/MS scanning in negative mode, using the 179.2 *m/z* fragment (the daughter ion of 12-HETE 319.2 *m/z*) for their detection.

In 6 out of 73 lipid extracts analysed in this section of the study, it was apparent that platelet activation had not occurred as there was no evidence of 12-HETE-PL generation. This occurred in 1 UBD patient, 3 DVT patients and 2 healthy controls. The UBD patient was re-called as this may have had implications with regards to the bleeding diagnosis and repeat testing confirmed the presence of six species of 12-HETE-PL on thrombin activation and repeat scanning by LC/MS/MS. The remaining samples (n=5) were excluded as HETE-PL had not been generated which was assumed to be due to experimental error with the activation process at the time.

6.2.2 Basal concentrations of 12-HETE-PL in UBDs, DVT patients and healthy controls

Basal concentrations of HETE-PL were detected in very small quantities and therefore, the most abundant positional isomer of 12-HETE-PE and -PC was used to examine whether there were any differences exhibited by platelets in the disease cohorts compared with healthy controls. Basal amounts of both PE(18:0a_12-HETE) and PC (18:0a_12-HETE) were similar in UBD patients compared to healthy controls, and were not increased in DVT patients. This differs from previous reports of elevated basal amounts of HETE-PE (platelets and neutrophils) in a thrombotic cohort of patients with antiphospholipid syndrome. It is notable however, that in 7 of the 12 antiphospholipid patients included, the unactivated platelets spontaneously aggregated during isolation. Here, despite the higher incidence of platelet aggregation in DVT patients compared with healthy controls as described in Chapter 3, samples were not analysed further in the event of visible clumping. It is possible that higher quantities of 12-HETE-PE may have been detected in the platelet isolates of these DVT patients if they had been analysed.

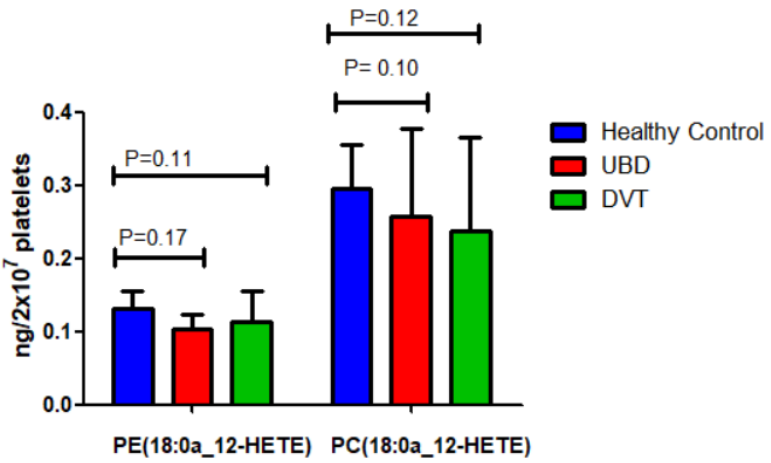


Figure 6.1 Basal concentrations of 12-HETE-PL are similar in healthy controls, UBDs and DVT patients Washed platelets were isolated from healthy controls, UBD and DVT patients and left unstimulated for up to 30 minutes. Basal concentrations of the most abundant 12-HETE positional isomer for PE and PC is shown. Platelet isolates which spontaneously aggregated during the isolation process were discarded. Healthy controls are depicted in blue, UBD patients in red and DVT patients in green. Data is shown in bar charts and expressed as mean basal concentration +/- S.E.

6.2.3 Generation of 12-HETE-PL by thrombin- activated platelets of UBD patients

In healthy controls and disease cohorts, all six molecular species of 12-HETE-PL were generated following thrombin activation displaying a similar pattern of distribution. There was a trend towards lower quantities of 12-HETE-PE in thrombin-activated platelets of UBD patients compared to healthy controls, although this did not reach statistical significance. Platelets from the Scott syndrome patient generated all four molecular species of 12-HETE-PE in comparable amounts to healthy controls and the UBD cohort as previously shown[134]. However, unlike the native externalised species of PE and PS, the amount of externalised 12-HETE-PE in the platelets of the Scott patient was not measured in this study. Therefore, conclusions about the contribution of 12-HETE-PE in supporting coagulation in the Scott patient cannot be inferred from this data.

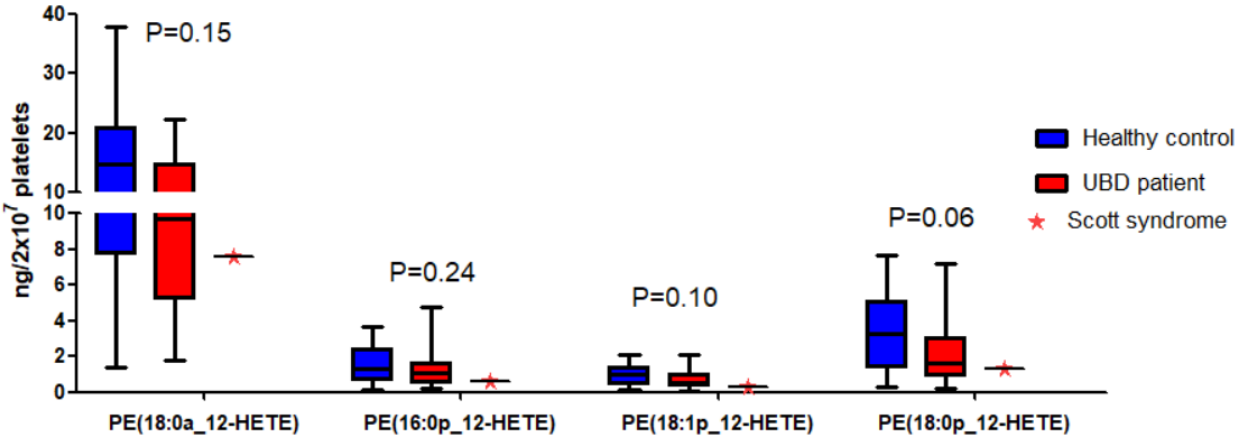


Figure 6.2a. A trend towards lower quantities of the four molecular species of 12-HETE-PE is shown in thrombin-activated platelets of UBD patients. Lipid extracts from washed human platelets were activated with 0.2 units/ml thrombin for 30 minutes at 37 ^{0}C and separated using LC/MS/MS with negative precursor scanning for 179.2 *m/z* (the daughter ion of 12-HETE 319.2 *m/z*) as described in Section 2.14, Chapter 2. The four molecular species of 12-HETE-PE (PE (16:0p_12-HETE), PE(18:1p_12-HETE), PE(18:0p_12-HETE) and PE(18:0a_12-HETE-PE)) generated in response to thrombin activation in platelets from healthy controls (blue) and UBD patients (red) is shown. Data from a patient with Scott syndrome is depicted by the red star. Data shown are median values (horizontal black bar), interquartile range (box) and maximum and minimum values (whiskers). P values were calculated using the Mann Whitney U test excluding the Scott patient.

Table 6.1. Descriptive data for 12-HETE-PE species

PE(18:0a_12-HETE) (ng/2 x 10^7 platelets)	UBD patients N=20	Healthy controls N=25	DVT patients N=20
Median	9.72	14.66	20.58
25th centile	5.19	7.71	10.90
75th centile	14.85	20.79	27.70
Mean	10.35	14.68	22.20
Std Deviation	5.81	9.46	12.73
Std Error	1.30	1.89	2.85

PE(16:0p_ 12 HETE) (ng/2 x 10^7 platelets)	UBD patients N=20	Healthy controls N=25	DVT patients N=20
Median	1.05	1.32	2.29
25th centile	0.49	0.71	1.69
75th centile	1.58	2.38	3.45
Mean	1.21	1.59	2.56
Std Deviation	1.01	1.59	1.54
Std Error	0.23	0.21	0.34

PE(18:1p_ 12 HETE) ng/2 x 10^7 platelets	UBD patients N=20	Healthy controls N=25	DVT patients N=20
Median	1.61	3.24	4.68
25th centile	0.91	1.42	3.32
75th centile	3.02	5.11	6.65
Mean	2.03	3.35	4.90
Std Deviation	1.59	1.59	2.52
Std Error	0.36	0.36	0.56

PE(18:0p 12 HETE) ng/2 x 10^7 platelets	UBD patients N=20	Healthy controls N=25	DVT patients N=20
Median	1.61	3.24	4.68
25th centile	0.91	1.42	3.32
75th centile	3.02	5.11	6.65
Mean	2.03	3.35	4.90
Std Deviation	1.59	1.59	2.52
Std Error	0.36	0.36	0.56

There was significantly less PC(18:0a_12-HETE) generated in the thrombin activated platelets in the UBD group (median IQR 9.5 ng/2 x 10^7 platelets (3.5-18.9)) compared to healthy controls (30.7 ng/2 x 10^7 platelets (13.8-41.8). This finding is of potential clinical interest since 12-HETE-PCs would be expected to be on the outer leaflet of the activated platelet where they can promote coagulation, and therefore reduced amounts could be associated with the bleeding that is observed in these patients. There were also somewhat lower quantities of PC(16:0a_12-HETE) but this was not statistically significant. Platelets from the Scott syndrome patient were shown to generate normal quantities of 12-HETE-PC and may explain why Scott platelets are able to support a low level of thrombin generation despite the reduced ability to externalise native PE and PS as previously reported [134].

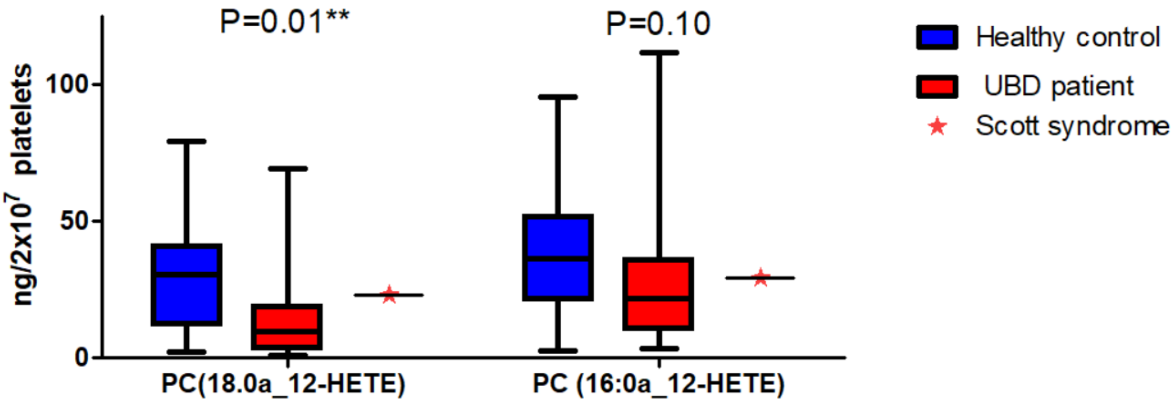


Figure 6.2b. A trend towards lower quantities of the two molecular species of 12-HETE-PC generation is shown in thrombin-activated platelets of UBD patients. Washed platelets from UBD patients, healthy controls and a patient were Scott syndrome were thrombin- activated and then the lipids were extracted and analysed to detect 12-HETE-PC species. The quantities of the two molecular species of 12-HETE-PC generated by activated platelets of healthy controls (blue) and UBD patients (red) is shown. Data from a patient with Scott syndrome is depicted by the red star. Data shown are median values (horizontal black bar), interquartile range (box) and maximum and minimum values (whiskers). P values were calculated using the Mann Whitney U test excluding the Scott patient.

Table 6.2. Descriptive data for 12-HETE-PC species

18:0a 12 HETE PC ng/2 x 10^7 platelets	UBD patients N=20	Healthy controls N=25	DVT patients N=20
Median	9.52	30.74	28.58
25th centile	3.50	13.84	18.54
75th centile	18.94	41.76	49.53
Mean	14.46	30.01	34.94
Std Deviation	15.80	19.13	20.16
Std Error	3.82	3.825	4.51

16:0a 12 HETE PC ng/2 x 10^7 platelets	UBD patients	Healthy controls	DVT patients
Median	21.81	42.15	39.10
25th centile	10.47	18.25	24.12
75th centile	35.63	51.84	79.23
Mean	30.17	39.60	57.12
Std Deviation	28.14	24.50	45.03
Std Error	6.293	4.900	10.07

6.2.4 Generation of 12-HETE-PL by thrombin-activated platelets of DVT patients

After the activation of platelets with thrombin, all four 12-HETE-PE species were increased in the thrombotic cohort compared with the healthy controls. This was statistically significant in for 3 of the species and borderline for one (Figure 5.3b). This may have potential implications as 12-HETE-PE supports increased thrombin generation and so may contribute to the prothrombotic phenotype of this cohort. It is not known whether the increased amounts of HETE-PE were causally associated with the thromboses or a consequence of the event as samples were taken at the time of the acute thrombotic episode.

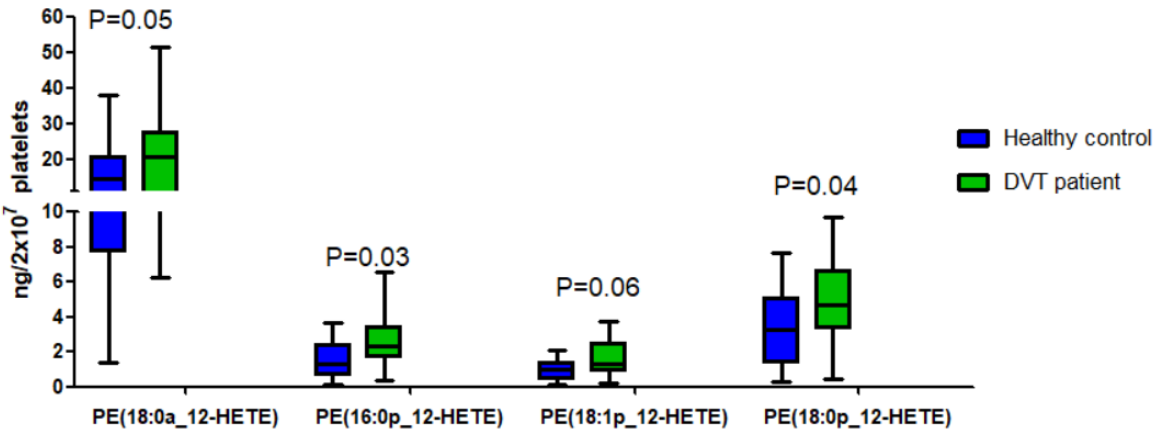


Figure 6.3a. Higher quantities of 12-HETE-PE species are generated by thrombin-activated platelets in DVT patients. Platelets were prepared and activated as described in The four molecular species of 12-HETE-PE (PE (16:0p_12-HETE), PE(18:1p_12-HETE), PE(18:0p_12-HETE) and PE(18:0a_12-HETE)) generated in response to activation with thrombin in platelets from healthy controls (blue) and DVT patients (blue) is shown. Data shown are median values (horizontal black bar), interquartile range (box) and values with maximum and minimum values (whiskers). P values were calculated using the Mann Whitney U test.

No differences were observed between the generation of 12-HETE-PC in the healthy controls and the DVT patients. This suggests that the platelets in the thrombotic cohort were not systemically activated and the increased amounts of HETE-PE observed could not be attributed to this potential mechanism.

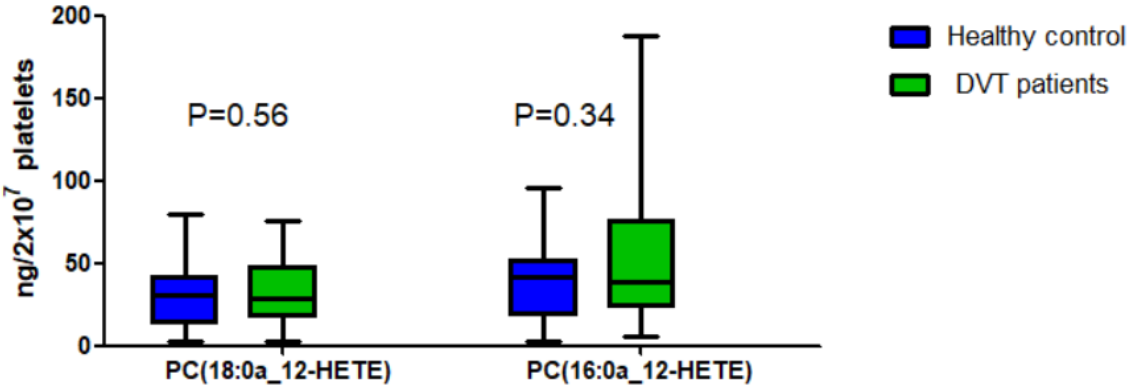


Figure 6.3b. Generation of 12-HETE-PC species in thrombin-activated platelets of healthy controls and DVT patients is similar. The two molecular species of 12-HETE-PC (PC (18:0a_12-HETE) and PC(16:0a_12-HETE) generated in response to activation with thrombin in platelets from healthy controls (blue) and DVT patients (green) is shown. Data shown are median values (horizontal black bar), interquartile range (box) and maximum and minimum values (whiskers). P values were calculated using the Mann Whitney U test.

6.3.1 Correlation of 12-HETE-PL with peak thrombin generation in UBD patients

The correlation between measurements of peak thrombin (derived from Chapter 4) and the most abundant species of 12-HETE-PL was examined. In healthy controls, no relationship was demonstrated between the amount of thrombin generation supported by washed platelets and the most abundant species of 12-HETE-PE and 12-HETE-PC generated after platelet activation. Similarly, no correlation was seen in the UBD cohort for 12-HETE-PE and 12-HETE-PC. The washed platelets in the thrombin generation assay were intrinsically activated instead of directly with an exogenous agonist, and therefore it is possible that this may have provided an insufficient stimulus for the complete generation of eoxPL which may account for the lack of correlation.

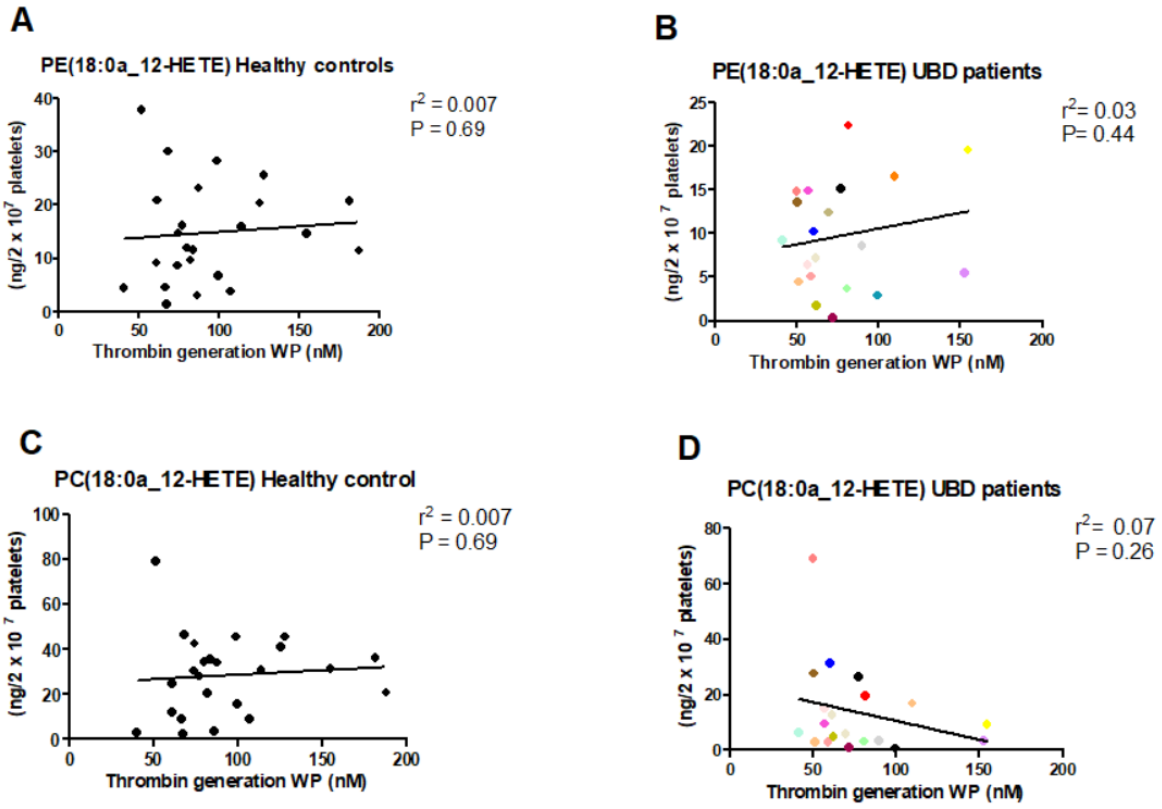


Figure 6.4 Correlation of 12-HETE-PL with peak thrombin generation supported by washed platelets in healthy controls and UBD patients. Thrombin generation supported by washed platelets was initiated by addition of a standardised liposome (0.5µM phospholipid/10pM TF) as described in Chapter 4. The correlation of peak thrombin measurements with the quantities of PE(18:0a_12-HETE) and PC(18:0_12-HETE) (ng/2 x 10^7 platelets) generated from thrombin-activated platelets is shown for both healthy controls (black) and UBD patients (coloured dots). For each correlation plot, the correlation co-efficient (r squared) is shown with corresponding p-values (P).

6.3.2 Correlation of 12-HETE-PL with peak thrombin generation in DVT patients

Finally, the correlation between peak thrombin and the 18:0/12-HETE positional isomer was also examined in the DVT cohort. As with healthy controls and UBD patients, no relationship was demonstrated between the amount of thrombin generated by washed platelets and the most abundant species of 12-HETE-PE and 12-HETE-PC. The same non-uniform pattern of clustering was observed between the 4 thrombotic outliers that were identified in the previous chapters and the rest of the DVT cohort.

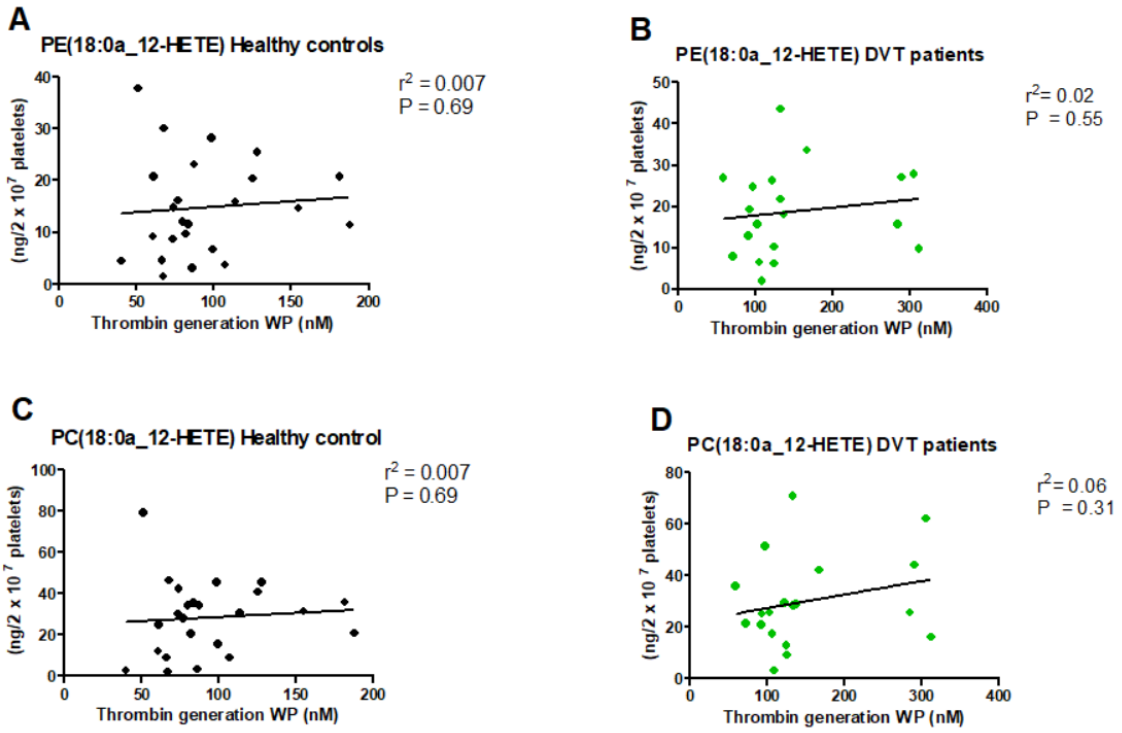


Fig 6.5 Correlation of 12-HETE-PL with peak thrombin generation supported by washed platelets in healthy controls and DVT patients. Washed platelets (1.5 x 10^8/ml) from healthy controls and DVT patients were added to liposomes and thrombin generation was measured as described in Chapter 4. The correlation with PE(18:0a_12-HETE) and PC(18:0a_12-HETE) is shown for both healthy controls (black) and DVT patients (green dots). For each scatter plot, the correlation co-efficient (r squared) is shown with the corresponding p-value (P).

6.4 Discussion

The aim of this chapter was to investigate whether there were differences in the amounts of HETE-PLs generated from platelets in patients with UBD and DVT. Overall patients with UBDs had a trend towards lower quantities of 12-HETE-PC and 12-HETE-PE whilst those with a thrombotic event had higher 12-HETE-PE but no change in 12-HETE-PC.

The reduction in 12-HETE-PC species in UBDs was statistically significant for PC(18:0a_12-HETE) and the median level was significantly lower than in the healthy control group. Incorporation of 12-HETE-PC into a membrane enhances thrombin generation and so the reduced amount found in the platelets of patients with UBDs would be anticipated to limit their ability to form clots in vivo. In addition, there was a trend towards low levels of PC(16:0a_12-HETE) and all species of 12-HETE-PE. It is possible that the combined effect of the reduced levels of these species might contribute to the ability of these platelets to support coagulation in vivo. Overall, the reduced levels of 12-HETE-PL species may explain at least in part, why the platelets of patients with UBDs supported lower peak levels of thrombin in the functional experiments despite externalising normal amounts of PS and PE. This may also explain the increased bleeding seen at the time of invasive procedures. It is interesting to note that patients with UBDs do not bleed spontaneously but only at the time of invasive procedures or haemostatic challenge such as menstruation. Similarly, mice lacking the ability to produce HETE-PE and HETE-PC only bleed after a haemostatic challenge such as tail transection which could be analogous to the operative procedures that were undergone by UBD patients recruited into this study [238].

Although there was a reproducible trend towards lower quantities of all 12-HETE-PE species in the UBD cohort this did not reach statistical significance. This may reflect the limited numbers of patients available to the study and the inherent wide variability of HETE-PE levels. The alternative explanation is that HETE-PE levels are not reduced in patients with UBD and this will need to be resolved by investigating further patients.

There was wide variation in the quantities of the six species of 12-HETE-PL generated by healthy donors and the disease cohorts. Previous studies have reported similar findings amongst platelets from donors with regards to the formation of HETE-PLs. For example, following activation with thrombin, levels of approximately 6 ng +/-1.42 ng/4 x 10^7 platelets (12-HETE-PE) and 18 +/- 4.61 ng/4 x 10^7 platelets (12-HETE-PC) were measured in 5 healthy donor platelets [134]. In another study, quantities of 12-HETE-PE in human platelets were reported to be in the range of 23 ng/4 x 10^7 platelets [146]. Here, the average quantity of PE(18:0a_12-HETE) in healthy controls was 14 ng/2 x 10^7 platelets (SE 1.8) and the average quantity of PC(18:0a_12-HETE) was 30 ng/2 x 10^7 platelets (SE 3.8).

Whilst 12-LOX mediates the oxidation of free fatty acids into 12-HETE, the formation of HETE-PL is a rapid process which is governed by a series of enzymatic reactions involved in the Lands cycle [141]. As described in Chapter 1, the hydrolysis of a Sn2 fatty acid by phospholipase A2 yields the fatty acid substrate which is available for oxidation by lipoxygenase. The resultant lysophospholipid is then re-esterified by acyl coA-ligases and lysophospholipid acyltransferases (LPLATs) or membrane bound O-acyl transferases (MBOATs) to re-form the specific phospholipid species [166, 167]. The substrate preference of these enzymes for different species of oxidised fatty acids however is not yet established in human platelets. As PC is the most abundant phospholipid in the platelet membrane, it would be anticipated that lyso-PC would be plentiful in supply as a substrate. It is therefore possible that reduced activity in an LPLAT or MBOAT enzyme that has greater specificity for the acylation of oxidised fatty acids into lyso-PC rather than lyso-PE could account for differences in the generation of 12-HETE-PC

and 12-HETE-PE that was shown here. However, at the current time this is unknown and future work will be required to inform this.

It is important to note, that whilst levels of 12-HETE-PC were lower overall in the UBD cohort, some of the patients generated levels comparable with healthy controls. This suggests that whilst some UBDs might be associated with reduced 12-HETE-PC, the group as a whole is heterogenous and the causes of bleeding may be attributable to more than one factor which may differ from one individual to another. A reduction in procoagulant 12-HETE-PC was observed in the whole group of UBD patients, demonstrating that reduced levels are present in proportion of this cohort of patients, other patients are likely to have different underlying causes for bleeding. This finding should be considered as hypothesis generating and would need to be reproduced in an independent group of UBD patients before any conclusion can be made regarding the potential contribution of 12-HETE-PC to the bleeding phenotype of these patients. This could potentially be a focus for future work and would require collaboration with other haemophilia centres.

In the patients with acute DVT, higher quantities of 12-HETE-PE species were observed compared to healthy controls and this was statistically significant for 3 species and borderline for the fourth. The amount of 12-HETE-PC was unchanged suggesting that the raised 12-HETE-PE levels were a specific finding and not simply due to systemic activation of platelets at the time of the thrombotic event. It has previously been shown that 12-HETE-PE enhances thrombin generation through the intrinsic tenase and prothrombinase reactions. The increased platelet HETE-PE found in this study may explain the ability of washed platelets from patients with venous thrombosis to support enhanced thrombin generation. In addition, they could support enhanced thrombin generation in vivo and hence could potentially contribute to the prothrombotic state. In animal studies, mice that are unable to make HETE-PEs are protected against an induced thrombosis [238]. It is possible, however that the thrombosis caused the increased HETE-PEs. In a recent study, increased HETE-PE were measured in circulating leucocytes and platelets of patients with a past history of thrombosis associated with the antiphospholipid syndrome [146] suggesting that the changes may persist. To investigate further the patients in this study could be retested to establish whether their HETE-PE levels are still raised.

Lastly, as changes in eoxPL were demonstrated in the experiments conducted in this chapter, their correlation with the peak thrombin measurements from Chapter 4 was investigated. It was hypothesised that lower quantities of 12-HETE-PC may contribute to the lower thrombin generation observed in the UBD patients and the higher quantities of 12-HETE-PEs may play a role in the higher thrombin generation seen in the DVT cohort. As described in Chapter 4, the platelets used in the thrombin generation assay were intrinsically activated whereas 12-HETE-PLs were generated following activation of platelets with thrombin. It is possible, that there was incomplete generation of 12-HETE-PL in the platelets used in the thrombin generation assay in the absence of an exogenous agonist.

In summary, the experiments in this chapter identified trends in lower quantities of 12-HETE-PE and 12-HETE-PC in UBD patients compared with healthy controls. Further studies in an independent cohort of UBD patients is required to confirm these findings. Higher quantities of 12-HETE-PE were demonstrated in the thrombotic cohort, supporting a potential role for these HETE-PL in promoting coagulation. These findings require further confirmation in larger studies.

Chapter 7- The effect of DDAVP on the thrombin generating capacity and phospholipid composition of the platelet membrane in patients with UBD

7.1 Introduction

Red cell transfusion due to intra-operative blood loss is common in everyday clinical practice. Anaemia following surgery is a recognised predictor of increased morbidity and mortality [242]. The transfusion of blood and its derivatives is associated with potential risks however, such as volume overload, blood group incompatibility, transfusion - related acute lung injury and transfusion transmitted infections [243].

Over the past decade, it has been shown that early administration of the antifibrinolytic drug tranexamic acid (TXA) improves survival of patients with major traumatic haemorrhage and is also of value in the setting of post-partum and surgical haemorrhage [244] [245]. In patients with acquired platelet dysfunction, for example those undergoing cardiopulmonary bypass, there is evidence to suggest that the haemostatic agent DDAVP is of value in reducing bleeding and transfusion requirements in the perioperative period [246]. Similarly, its use is advocated in the setting of excessive bleeding and platelet dysfunction due to uraemia [188, 247].

DDAVP is an established treatment in patients with congenital defects of platelet function and is recommended in guidelines for the bleeding associated with acquired platelet dysfunction [188] [247]. By increasing von Willebrand factor release and FVIII, the mechanism of action of DDAVP in the treatment of sub-types of von Willebrand disease and mild haemophilia A is well established. Its mechanism of action in managing bleeding in patients with platelet dysfunction is not well established but it has recently been attributed to its potential role in increasing procoagulant platelets [51].

It is reported that up to 88% of patients with UBD experience bleeding at the time of surgery. In a study of 33 UBD patients, 18 patients in the cohort (54.5%) had received red cell transfusion and other blood products including fresh frozen plasma (FFP) and cryoprecipitate following haemostatic challenges. It has recently been shown that TXA either alone or in combination with DDAVP is an effective management strategy for UBD patients undergoing surgery. This regimen was reported to have efficacy in 90% of patients undergoing both minor and major invasive procedures, although the exact mechanism of action of DDAVP in this cohort of patients is unknown [184].

The aim of this chapter was to investigate the effect of DDAVP on externalised species of PE and PS in UBD patients undergoing a haemostatic procedure. In addition, the effect of DDAVP on the generation of eoxPL and thrombin generation supported by washed platelets was also examined.

7.2 Demographics and clinical details of UBD patients receiving DDAVP

Three patients diagnosed with UBD and 1 patient with a platelet function defect (PFD) were admitted for a planned elective invasive procedure and were therefore recruited to this section of the study. The diagnosis of UBD was made in 3 patients in accordance with criteria outlined in Chapter 2. The remaining patient had been diagnosed with a PFD following presentation with easy bruising and had a reproducible absent response to adrenaline on platelet aggregometry testing. The patient demographics, bleeding score and details of previous haemostatic challenges are shown in Table 7.1.

Table 7.1 Details of bleeding score and previous haemostatic challenges in UBD patients undergoing a haemostatic challenge.

Age/gender	Diagnosis	ISTH BAT Bleeding score	Previous haemostatic challenge (agent)	Clinical outcome of previous treatment
58 F	UBD	11	Endometrial ablation & insertion of a mirena coil (*DDAVP & TXA)*	No bleeding
56 F	UBD	11	Discectomy (*DDAVP & TXA)* Bunion removal (*DDAVP &TXA*)	No bleeding
67F	UBD	6	Shoulder arthroscopy x 3 (*DDAVP & TXA)* Bunion removal (*DDAVP &TXA*)	No bleeding
54F	PFD	3	3 x dental extraction (*TXA)*	No bleeding

(UBD = Unclassified bleeding disorder; PFD = Platelet function disorder; DDAVP = Desmopressin; TXA = Tranexamic acid; F = Female, ISTH BAT = International Society of Thrombosis and Haemostasis Bleeding Assessment Tool)

A blood draw (in the absence of DDAVP) was taken from each patient and the washed platelets were isolated as outlined in Chapter 2. Baseline thrombin generation supported by washed platelets was measured and phospholipids were extracted from thrombin-activated platelets using the experimental methods detailed previously. DDAVP was administered to all patients either intravenously (IV) or subcutaneously (SC) at a dose of 0.3 µg/kg prior to the haemostatic procedure. A further blood draw was taken 1.5- 2 hours following drug administration and both thrombin generation and phospholipid extraction were re-performed. Details and clinical outcome of the procedure are shown in Table 7.2.

Table 7.2 Details and clinical outcome of haemostatic procedure in UBD patients receiving DDAVP

Patient	Diagnosis	Drug/route	Procedure	Clinical outcome
1	UBD	DDAVP SC	Dental extraction	No bleeding
2	UBD	DDAVP IV	Fundoplication & banding of haemorrhoids	No bleeding
3	UBD	DDAVP SC	Excision of skin lesion	No bleeding
4	PFD	DDAVP IV	Bowel resection	No bleeding

(UBD = Unclassified bleeding disorder; PFD = Platelet function disorder; DDAVP = Desmopressin SC = Subcutaneous; IV = intravenous)

7.3 Results

7.3.1 The effect of DDAVP on thrombin generation in UBD patients

Thrombin generation supported by washed platelets was measured before and after DDAVP. In all 3 UBD patients an increase in peak thrombin generation and velocity index was demonstrated after drug administration. This was not statistically significant for the group probably due to insufficient numbers (Figure 7.1). There was insufficient time to recruit more patients because they could only be tested at the time of an invasive procedure when DDAVP was due to be administered. The peak thrombin generation for the 3 UBD patients at baseline (before DDAVP) was in a range of 50-73.5 nM which is low compared to healthy controls but comparable to other UBD patients as shown previously (Figure 4.4 and Table 4.1). No change in these parameters occurred in the patient with PFD apart from a shortening in the lag time. DDAVP had no effect on the ETP or the lag time in the UBD patients.

Patients 1 and 3 were treated with subcutaneous DDAVP and their peak thrombin generation increased 1.3 and 1.5 fold, respectively. Patient 2 was treated with intravenous DDAVP and their peak thrombin generation increased 2.2 fold suggesting that this mode of administration was associated with a larger effect of thrombin generation. However, more patients will need to be studied to establish whether there is a reproducible association between mode of administration of DDAVP and effect on thrombin generation.

Two of the 3 UBD patients (Patient 1 and Patient 2) had been recruited previously. Their peak thrombin generation on initial testing and subsequently during investigation of the effect of DDAVP are shown in Table 7.3. The baseline thrombin generation of the two patients was reproducibly low compared to healthy controls demonstrating that the finding was consistent over time.

Table 7.3 Peak measurements of thrombin at baseline and following DDAVP

Peak TG (UBD range:- 56.93-87.68nM)	Baseline peak TG (nM)	Baseline peak TG (nM) (pre-DDAVP)	Peak TG (nM) (post-DDAVP)
Date of sample collection	Autumn 2017	Spring 2019	Spring 2019
Patient 1	80.71	50.00	65.72
Patient 2	56.85	73.50	157.86
Patient 3	----	64.04	93.00

(UBD= unclassified bleeding disorder; DDAVP = desmopressin; TG = thrombin generation)

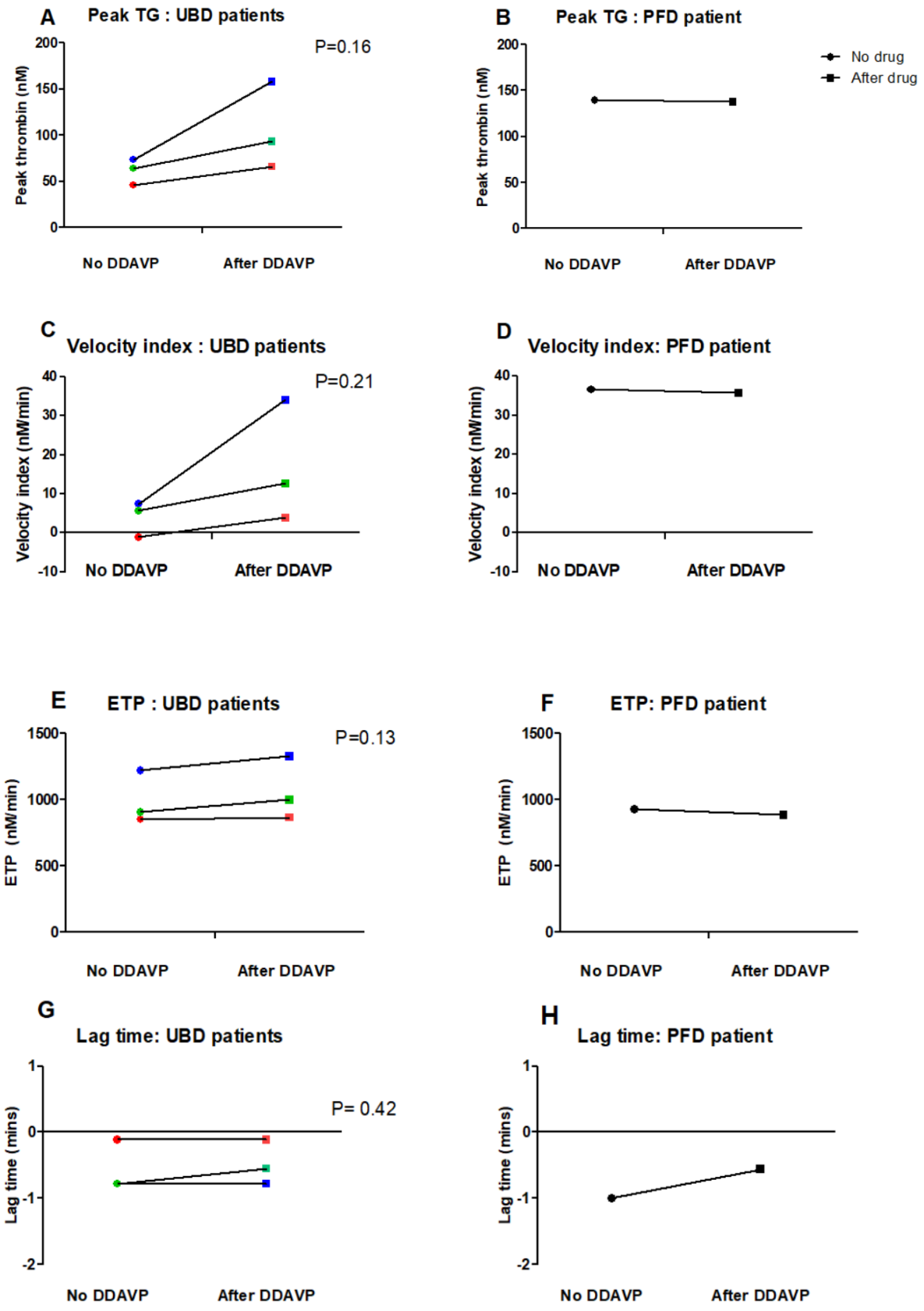

A
Peak TG : UBD patients
P=0.16
Peak thrombin (nM)
200
150
100
50
0
No DDAVP
After DDAVP
B
Peak TG : PFD patient
No drug
After drug
Peak thrombin (nM)
200
150
100
50
0
No DDAVP
After DDAVP
C
Velocity index : UBD patients
P=0.21
Velocity index (nM/min)
40
30
20
10
0
-10
No DDAVP
After DDAVP
D
Velocity index: PFD patient
Velocity index (nM/min)
40
30
20
10
0
-10
No DDAVP
After DDAVP
E
ETP : UBD patients
P=0.13
ETP (nM/min)
1500
1000
500
0
No DDAVP
After DDAVP
F
ETP: PFD patient
ETP (nM/min)
1500
1000
500
0
No DDAVP
After DDAVP
G
Lag time: UBD patients
P= 0.42
Lag time (mins)
1
0
-1
-2
No DDAVP
After DDAVP
H
Lag time: PFD patient
Lag time (mins)
1
0
-1
-2
No DDAVP
After DDAVP

Fig 7.1 Trends towards increased measurements of peak thrombin and velocity index are shown in UBD patients after DDAVP infusion. Baseline measurements of peak thrombin generation, velocity index, lag time and ETP are shown for UBD patients (n=3) and a patient with PFD (n=1). Patient 1, 2 and 3 are represented by red, blue and green coloured shapes (dots and squares) respectively. Patient 4 is the single patient with PFD and is represented by black dots and squares. The P values were calculated using the Wilcoxon matched pairs test (2 tailed).

7.3.2 The effect of DDAVP on externalised PE and PS in UBD patients

Using methods described in Chapter 2, washed platelets were activated with thrombin and the externalised lipids were extracted and quantitated using LC/MS/MS. In all 3 UBD patients, there was a statistically significant increase in externalisation of all species of PS ($P< 0.05$) after DDAVP. A trend towards an increase in individual PE species was observed in the patient with PFD and the 3 UBD patients, although this did not reach statistical significance. These trends were consistent for each species of lipid as shown in Figure 7.2.

The amounts of species of PS and PE at initial testing when first enrolled and subsequently tested during investigation of the effect of DDAVP are shown in Table 7.3 for Patient 1 and Patient 2 who were recruited previously. The results show that the levels were broadly reproducible.

Table 7.4 Measurements of externalised PE/PS species at baseline and following DDAVP

Patient 1	**Baseline (1st time) Ext aPL ng/4x10^{7}platelets**	**Baseline (pre-DDAVP) Ext aPL ng/4x10^{7}platelets**	**Post DDAVP Ext aPL ng/4x10^{7}platelets**
Date of sample collection	Autumn 2017	Spring 2019	Spring 2019
DOPS	1.71	1.42	2.79
SOPS	5.61	3.50	6.91
SAPS	7.25	5.37	11.07
PpAPE	11.00	9.66	14.32
SOPE	1.91	1.18	2.04
SpAPE	20.8	49.5	47.67
SAPE	18.7	11.60	16.87

Patient 2	**Baseline (1st time) Ext aPL ng/4x10^{7}platelets**	**Baseline (pre-DDAVP) Ext aPL ng/4x10^{7}platelets**	**Post DDAVP Ext aPL ng/4x10^{7}platelets**
Date of sample collection	Autumn 2017	Spring 2019	Spring 2019
DOPS	0.73	0.53	2.06
SOPS	3.11	1.74	6.91
SAPS	3.92	2.65	10.71
PpAPE	4.44	4.58	26.25
SOPE	1.7	0.52	2.04
SpAPE	13.9	24.29	112.59
SAPE	5.9	6.11	26.46

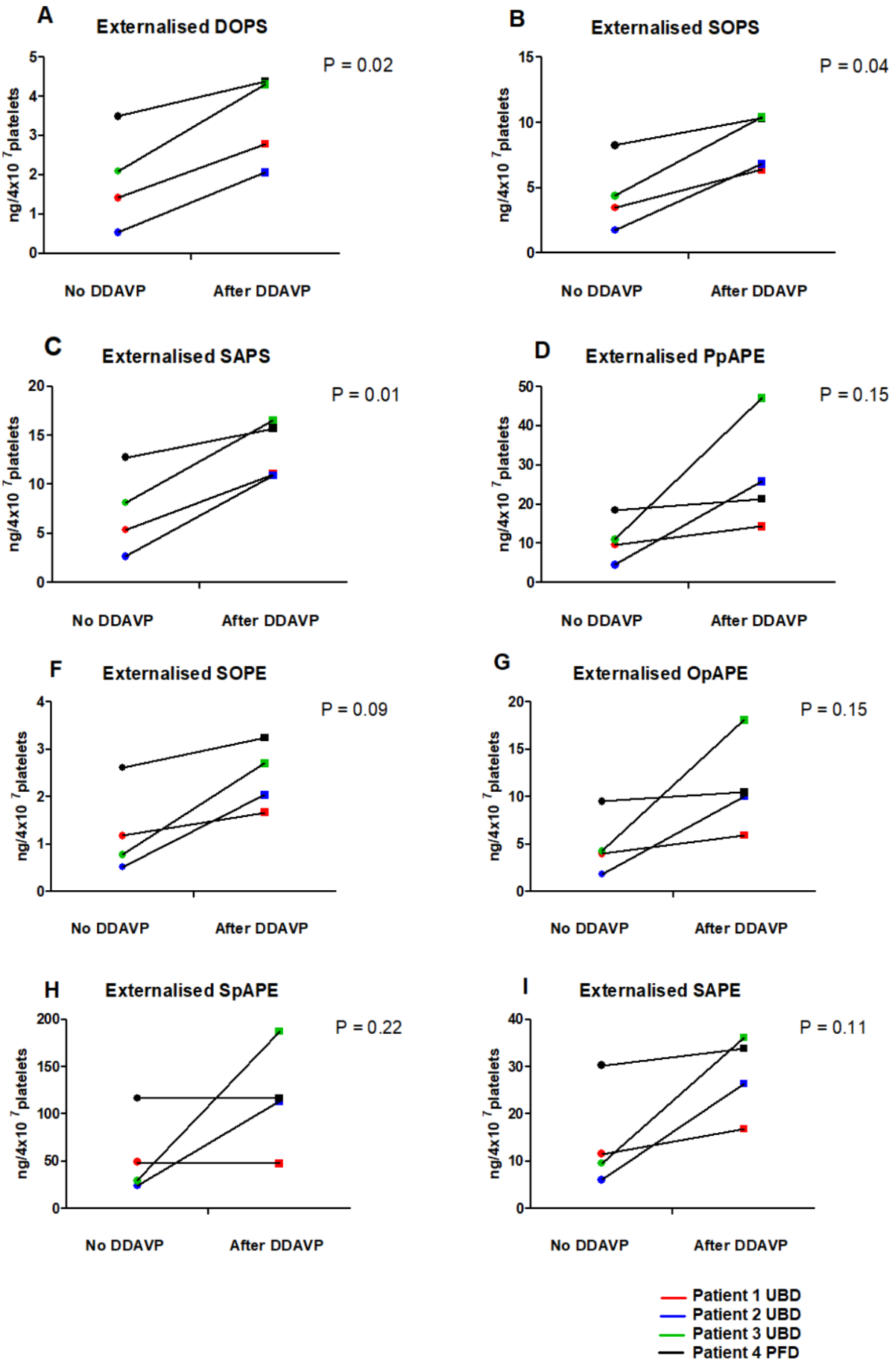

A
Externalised DOPS
P = 0.02
ng/4x10 7platelets
No DDAVP
After DDAVP
B
Externalised SOPS
P = 0.04
C
Externalised SAPS
P = 0.01
D
Externalised PpAPE
P = 0.15
F
Externalised SOPE
P = 0.09
G
Externalised OpAPE
P = 0.15
H
Externalised SpAPE
P = 0.22
I
Externalised SAPE
P = 0.11
Patient 1 UBD
Patient 2 UBD
Patient 3 UBD
Patient 4 PFD

Figure 7.2 Externalisation of PS species is increased in UBD patients after DDAVP infusion. Measurements of each species of PE and PS is shown for the UBD patients (n=3) and the patient with PFD (n=1) at baseline and following administration of DDAVP. P values for the UBD cohort were calculated using the Wilcoxon matched pairs test (two-sided) with the exclusion of the patient with PFD.

7.3.3 The effect of DDAVP on 12-HETE-PL in UBD patients

Lastly, to evaluate whether DDAVP would have an influence on the generation of eox PL, lipid extracts from thrombin-activated washed platelets were fragmented by LC-MS/MS and the six 12-HETE-PE and -PC species were identified using the 12-HETE daughter ion *m/z* 179.2. A high degree of variability was observed amongst the 4 patients. There was no clear pattern evident in terms of the response to treatment with DDAVP (Figure 7.3) although there was a trend for HETE-PE to increase. Further patients need to be investigated to address this question.

The results of HETE-PL generated from thrombin-activated platelets at baseline and subsequently following DDAVP are shown in Table 7.4 for Patient 1 and Patient 2 who were recruited previously.

Table 7.5 Generation of 12-HETE-PL at baseline and following DDAVP

Patient 1	**Baseline (1st time) 12-HETE-PL ng/2x10^{7}platelets**	**Baseline (pre-DDAVP) 12-HETE-PL ng/2x10^{7}platelets**	**Post DDAVP 12-HETE-PL ng/2x10^{7}platelets**
Date of sample collection	Autumn 2017	Spring 2019	Spring 2019
PE(18:0a_12-HETE)	19.55	20.47	20.35
PE(16:0p_12-HETE)	1.32	1.83	2.13
PE(18:1p_12-HETE)	0.91	0.79	1.14
PE(18.0p_12-HETE)	3.22	3.88	4.92
PC(18:0a_12-HETE)	9.44	40.58	30.59
PC(16:0a_12-HETE)	20.00	60.44	36.30

Patient 2	**Baseline (1st time) 12-HETE-PL ng/2x10^{7}platelets**	**Baseline (pre-DDAVP) 12-HETE-PL ng/2x10^{7}platelets**	**Post DDAVP 12-HETE-PL ng/2x10^{7}platelets**
Date of sample collection	**Autumn 2017**	**Spring 2019**	**Spring 2019**
PE(18:0a_12-HETE)	6.41	8.77	20.35
PE(16:0p_12-HETE)	0.79	0.81	3.96
PE(18:1p_12-HETE)	0.35	0.29	1.90
PE(18.0p_12-HETE)	1.36	1.75	9.33
PC(18:0a_12-HETE)	15.07	19.50	29.95
PC(16:0a_12-HETE)	31.07	27.12	29.31

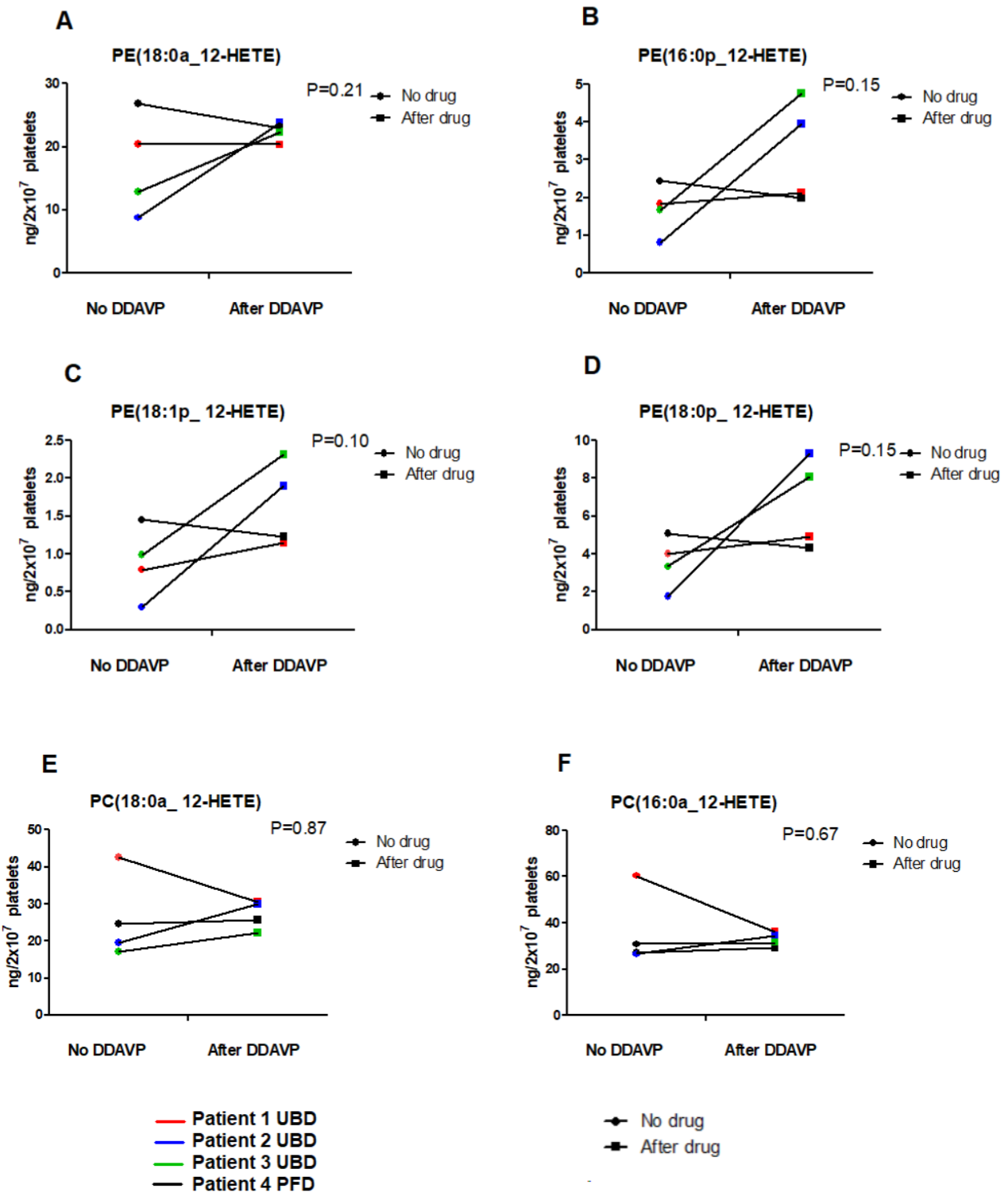


Figure 7.3. DDAVP has a variable effect on the generation of 12-HETE-PL species in UBD patients. Measurements of each species of 12-HETE- PE and 12-HETE-PC is shown for the UBD patients (n=3) and the patient with PFD (n=1) at baseline and following administration of DDAVP. P values for the UBD cohort were calculated using the Wilcoxon matched pairs test (two-sided) with the exclusion of the patient with PFD.

7.4 Discussion

In this chapter, the effect of DDAVP on the thrombin generating-capacity of washed platelets in UBD patients was investigated. In addition, its effect on the externalisation of PE/PS species and generation of eoxPL in this patient cohort was also explored.

Following DDAVP infusion, an increase in peak thrombin was demonstrated in all 3 patients with UBD although this did not reach statistical significance. Recruitment was limited by the small number of UBD patients undergoing a haemostatic procedure during the study period. Using these data to inform a power calculation (using an alpha of 0.05 and a power of 0.9), a sample size of 8 UBD patients would be required to identify whether DDAVP has a significant effect on thrombin generation.

The re-testing of the two UBD patients on two separate occasions was associated with reproducibly low results but variability over time was seen. This is reflected in the high co-efficient of variance (CV 40%) of the thrombin generation assay, and the significant overlap between peak thrombin measurements in the UBD cohort and healthy controls as observed in Chapter 6. As a result, there will be a wide 'acceptable' range defining 'normal' peak thrombin generation (68-121 nM) which in this assay overlaps with that of the UBD cohort (56-87nM). This suggests that the thrombin generation assay as currently set up is imprecise for diagnostic purposes but does appear to demonstrate that people with UBDs as a group have a reproducibly reduced ability to support thrombin generation.

An increase in thrombin generation was observed in all 3 patients after DDAVP, however only in Patient 2 was the concentration above the normal range. Patient 2 was the only patient to receive intravenous DDAVP and further work needs to assess whether this is a chance finding or of potential clinical importance. Of note, the peak thrombin generation in this patient after DDAVP was comparable with the DVT cohort (97-151 nM)

The quantities of externalised PE and PS in patients 1 and 2 were largely consistent over time (Table 7.4). In contrast, wide variability was observed in the generation of eoxPL, which suggests that their formation although enzymatically controlled, is a far more dynamic and temporal process than that which governs the externalisation of native PE/PS (Table 7.5). It may however be too simplistic to expect a high degree of homogeneity between the assays of these patients at two very different time points. Specifically, biological factors such as dietary intake, blood levels of lipid and stress hormones in the peri-operative or peri-procedural period may be influential as well as the diurnal and seasonal effects on fatty acid metabolism [248-250].

Although no direct comparisons can be made for UBD patients, notable observations can be drawn from a study investigating the effect of DDAVP in patients with platelet dysfunction. In 38 PFD patients, an increase in thrombin- generating rate was measured following DDAVP infusion using platelet rich plasma and gel filtered platelets reconstituted in FV deficient plasma. Furthermore, these patients had generated an increase in PS-exposing procoagulant platelets as measured by annexin V binding following DDAVP infusion. The platelets of these PFD patients were activated with convulxin and thrombin prior to measurements of both thrombin generation and flow cytometry to promote the generation of procoagulant or 'collagen and thrombin-activated (COAT) platelets.' It is therefore likely that more significant differences in peak thrombin generation following DDAVP would have been demonstrated in the UBD patients described here, if the platelets had been dually activated with these agonists rather than relying on their intrinsic activation induced by the initial thrombin produced in the assay by the TF-bearing liposomes.

Consistent with the findings of the study above, the 3 UBD patients externalised increased quantities of PS following DDAVP infusion and this was also seen in the patient with PFD. A trend towards increased PE species was also observed in all patients but further subjects will need to be studies to establish whether this is a statistically significant finding. It is reported that at least 5% of patients with PFD do not respond to the effect of DDAVP in terms of enhancing COAT generation as detected by annexin V binding. In addition, the generation of procoagulant platelets in responding patients is reported to continue to increase 2 hours post-DDAVP peaking at 4 hours after administration. In the studies reported here samples were taken 1.5 hours after DDAVP and despite mass spectrometry being a more sensitive method in detecting PE and PS, it is possible that further increases may have been observed if measurements had been taken at these later time points. Although DDAVP appeared to have a procoagulant effect in terms of promoting PE/PS externalisation in the 3 UBD patients, it is possible a proportion of non-responders may exist as reported in PFD patients.

In UBD patients, it is known that DDAVP reduces the risk of bleeding at the time of surgery and is effective treatment in this group of patients. Within the limitations of the assay, the finding that peak thrombin generation was higher in all 3 UBD patients following DDAVP compared with the baseline value at the same time-point, coupled with increased externalised PE/PS provides insights into a potential mechanism for its clinical efficacy in UBD patients. This raises the possibility that one of the mechanisms of action of DDAVP to prevent abnormal bleeding at the time of surgery may be related to increased thrombin generation due to PS/PE externalisation on the surface of platelets. More data are required to test this hypothesis.

The mechanism of action of DDAVP in enhancing the ability to form procoagulant platelets has been associated with increased platelet intracellular fluxes of Na^+ and Ca^{2+}. It is proposed that Na^+ entry may occur from activation of non-selective cation channels which interact with Na^+/Ca^{2+} exchangers, thereby increasing the intracellular Ca^{2+} that is necessary for PS/PE exposure, particularly when dually activated by collagen and thrombin [251]. It is notable that the platelets used in the phospholipid assays of this study were solely activated with thrombin. It is possible further differences between pre and post DDAVP samples may have been demonstrated following activation of platelets with both agonists.

It is anticipated that following the generation of eoxPL by activated platelets, the subsequent exposure of 12-HETE-PE may parallel that of PS although it is not established whether this process occurs by independent mechanisms. As 12-HETE-PCs are already located on the external leaflet of the platelet membrane, it may also be hypothesised that DDAVP may enhance their generation. Within the limitations of this data set, observations of such trends or alternatively a counter-effect with native lipids were not apparent.

In summary, an increase in peak thrombin generation was observed in all 3 UBD patients following the administration of DDAVP. An increase in PE/PS externalisation in the 3 UBD patients was demonstrated although no consistent effect was evident with regards to the generation of 12-HETE-PL. The increased externalisation of platelet PS/PE by DDAVP may explain why DDAVP is a useful treatment in this situation. Larger studies on the effect of DDAVP in UBD patients are required to confirm these findings and investigate their clinical significance.

Chapter 8 - General discussion

The main aim of this thesis was to investigate whether the phospholipid surface of the platelet membrane is implicated in bleeding disorders of unknown cause and in venous thrombosis. This was investigated using a combination of functional assays tailored to be sensitive to the platelet surface and a lipidomics approach specifically focusing on native PE/PS externalised by platelets and eoxPL generated following agonist activation. It is now widely acknowledged that APL and eoxPL are procoagulant classes of lipids based on in vitro research demonstrating that these specific species of lipids enhance coagulation by increasing thrombin generation through the tissue factor FVIIa, tenase and prothrombinase complexes [146, 149]. Furthermore, platelet phospholipid dysregulation and resultant haemostatic failure in humans is best exemplified by the exceptionally rare bleeding disorder Scott syndrome. In this disorder, platelets are able to externalise only very limited amounts of PE and PS and severe bleeding occurs at the time of invasive procedures [134, 155]

The first aim of the thesis was to assess the ability of the platelet phospholipid surface to support thrombin generation in patients with UBD and in individuals with acute DVT. The results show that the platelets of patients with UBD have reduced ability to support thrombin generation, which may in part explain their bleeding phenotype. The thrombin generation assay was designed to be sensitive to the platelet phospholipid surface, hence a minimal amount of phospholipid (0.5 μM) was used in the TF- bearing liposomes in order to increase the sensitivity of the assay to the lipid composition of the platelet membrane.

In a recent study by Thomas et al, a standard phospholipid composition of 4 μM was used to trigger coagulation and there was no difference in the amount of thrombin generated from the platelet poor plasma of UBD patients and healthy controls [185]. This study did not investigate the patients' platelets and used excess phospholipid to make the assay sensitive to coagulation factors in the plasma. This demonstrates the importance of the phospholipid composition in haemostatic assays and further implicates the phospholipid composition of the platelet membrane as having an influence on the underlying pathophysiology of this disorder. Despite the lower peak thrombin measurements and velocity index in the cohort of UBDs demonstrated in Chapter 4 of this thesis, there was significant overlap in these parameters with the ranges obtained from healthy controls. The washed platelet thrombin generation assay as described in this thesis therefore cannot be used for the diagnosis of UBD in the clinical setting but provides some insight into the global pathology underlying UBDs. However, a more sensitive assay might be developed by adjusting the TF and phospholipid composition of the triggering liposomes or using FXa/FVa cleavage of FII as the read- out rather than pooled plasma.

Higher peak thrombin generation and velocity index were demonstrated in thrombosis patients, however whilst this may suggest an increased ability of the platelet surface to support thrombin generation a degree of caution with regards to over-interpretation is required due to the increased platelet hyper-reactivity observed from samples collected from these individuals. Although moderate correlations of peak thrombin generation with APL externalisation were observed in the healthy controls and UBD patients, changes in externalised PS/PE could not explain the increased peak thrombin generation because this was shown to be the same in the two groups. The changes in thrombin generation might more accurately reflect changes in eoxPL that were observed in the two cohorts of patients in Chapter 6. The lack of correlation between measurements of peak thrombin generation and eoxPL for individual study participants could be due to the intrinsic activation of platelets in the assay rather than exogenously with thrombin for example.

Additionally, it was shown that the microvesicles of UBD patients had reduced ability to support thrombin generation which may be an additional risk factor for the bleeding observed in UBD patients. As discussed previously, these microvesicles were derived from the supernatant of the individuals' platelet poor plasma and were therefore not only platelet-derived but are likely to also have been generated from white cells or endothelial cells. The microvesicles isolated and tested in this thesis were representative of resident microvesicles in the patients' plasma in the 'resting state'. The thrombin generation supported by the microvesicles of DVT patients was not increased compared to healthy controls, which differs from reports of their elevation in cancer associated thrombosis and their proposed role in promoting arterial and venous thrombosis [205, 234]. It is possible that isolating platelet-derived microvesicles following activation with an agonist may have demonstrated differences in this regard. Further in-depth investigation of platelet-derived microvesicles in UBDs and thrombosis patients is required in a specialist laboratory using ISEV recommendations for their isolation and detection. The profiling of APL externalisation and eoxPL in platelet microvesicles was not carried out in this thesis and will require further investigation.

The results presented in Chapter 5 did not identify a reduction in APL externalisation in UBDs or alternatively an increase in their synthesis in cases of DVT with no known inherited or acquired thrombophilia. These observations demonstrate that whilst thrombin generation on washed platelets is decreased in UBDs and increased in patients with venous thrombosis, this cannot be attributed to changes in externalised APL. The techniques to investigate phospholipids in platelets are specialised and outside the remit of a specialist haemostasis laboratory. Possible screening tests include annexin V binding and prothrombin consumption index but these are not routine practice and disorders associated with abnormalities of these would therefore remain undiagnosed. As outlined in Chapter 5, analysing samples using the biotin assay over a prolonged study period had experimental limitations which require further consideration for other studies. One possible approach would be to extract and analyse samples at the same time, although dependent on the number of samples involved this may prove highly labour intensive.

Although a small number of studies have reported on increased APL externalisation in prothrombotic conditions it is important to note that these were investigated using flow cytometry rather than mass spectrometry methods and were in association with other pathologies such as malignancy and myeloproliferative neoplasms in contrast to the thrombotic cohort investigated here. It is therefore possible that other associated procoagulant factors such as TF and TF-bearing cells may have been influential**.**

The experiments in Chapter 6 suggest eoxPL rapidly generated by activated platelets may contribute to the bleeding phenotype observed in UBDs. A significant reduction in procoagulant 12-HETE-PC was demonstrated in the UBD cohort compared with healthy controls. The trends towards lower quantities of 12-HETE-PE generated by the platelets of UBD patients were not as clear cut as those of 12-HETE-PC and this may be due to the small study population. The finding may also implicate a defect in the synthesis or remodelling pathway of the different species of 12-HETE-PL. To date, the genetic defect in UBDs is unknown and there may be multiple minor defects rather than a single major abnormality. As discussed in Chapter 3, 19 UBD patients described in this thesis were enrolled into BRIDGE-BPD, and only two had a causative mutation identified. These were a variant in the gene encoding the actin cytoskeletal regulator tropomyosin 4 (TPM4) of megakaryocytes and platelets and a variant in the MYH9 gene [228]. Another study recently reported on an absence of genetic pathogenic variants in 45 UBD patients who underwent investigation with thrombogenomics [185]. The results of BRIDGE-BPD showed that of 619 patients enrolled with

UBDs, a mutation was only identified in 3.2%. BRIDGE-BPD selected a panel of 87 coagulation genes and platelet genes associated with bleeding and the very low yield suggests that mutations in the genes tested are very unlikely to be implicated[7]. Investigating whether mutations in genes implicated in the synthesis and remodelling of 12-HETE-PL in this patient cohort would be interesting. The observations of reduced 12-HETE-PC that were shown in this thesis warrant investigation in a large independent cohort of UBD patients to validate these findings and establish whether they are reproducible.

Consistent with a study of thrombotic patients with antiphospholipid syndrome, increased quantities of 12-HETE-PE were demonstrated in the DVT cohort in this thesis as shown in the experiments concluding Chapter 5 [146]. Interestingly, quantities of 12-HETE-PC generated by activated platelets were similar with those measured in healthy controls, suggesting that systemic activation of the platelet did not occur at the time of the thrombotic event and that the increased 12-HETE-PE cannot be explained by this mechanism. It remains unknown whether the increased synthesis of 12-HETE-PE was contributory to the thrombotic event or a resultant feature and repeat testing of these individuals outside of the acute setting would be required for clarification.

Lastly the effect of DDAVP, the haemostatic agent used empirically in UBDs was investigated in Chapter 7. Interestingly, trends towards higher peak thrombin generation and velocity index were observed following DDAVP administration in 3 patients with UBD undergoing a haemostatic challenge. Furthermore, this was associated with an increase in externalisation of all species of PS ($P<0.05$). This is consistent with the work of Colucci et al, who proposed a novel mechanism of action of DDAVP in selectively enhancing the role of procoagulant platelets [51]. Whilst this was demonstrated in patients with platelet function defects using annexin V binding, the experiments in this chapter confirmed these finding in UBD patients using LC/MS/MS to profile the APL species. This provides interesting insights into the underlying mechanisms of DDAVP in platelet disorders and UBDs. Increases in 12-HETE-PL in contrast were not uniform or significant, however experiments were limited by the small number of UBD patients undergoing a haemostatic challenge at the time of recruitment. Further studies are required in a larger independent cohort of UBD patients to confirm and expand these findings.

This thesis has raised some areas requiring further work. Firstly, a larger study confirming the findings of eoxPL is required in an independent cohort of UBD patients. If validated, this needs to be combined with genomic studies looking for potential variants in genes coding for enzymes involved in the phospholipid remodelling pathway. The question also arises whether an increase in procoagulant eoxPL occurs in other thrombotic disorders for example in arterial disorders such as acute coronary syndrome and whether their increased generation is an independent risk factor for a thrombotic event or is associated with a recurrent episode. The elevation of eoxPL in venous thrombotic disease requires investigation following the acute thrombotic event to fully elucidate their contribution in enhancing thrombin generation. In addition, the role of APL in other thrombotic disorders requires clarification. For example, does an increased externalisation of APL occur in atherosclerosis or other acquired thrombotic disorders such as cancer-associated venous thrombosis. The role of DDAVP in increasing APL externalisation in UBD and other platelet disorders needs validating in a large study, using specialised LC/MS/MS techniques for in-depth analysis. Furthermore, the role of other lipids in the platelet membrane, specifically sphingolipids and cholesterol require future research in disorders of haemostasis and thrombosis.

Finally, whilst the thrombin generation assay was able to demonstrate global changes in the phospholipid platelet membrane for example in each of the disease cohorts versus healthy controls and following the administration of DDAVP, it could not be used for diagnostic purposes. Whilst high donor intra-variation is inevitable despite the standardisation of plasma and liposome composition, this assay requires further refinement to further assess the ability of the thrombin generation assay in differentiating patients with UBD in order to increase its clinical utility. In attempting to mirror the coagulation reactions at the level of the platelet phospholipid surface, it is evident that multiple variables were introduced which compromised the precision of the assay.

In summary, the results of the experiments conducted in this thesis support the original hypothesis that the platelet phospholipid membrane may be implicated in patients with UBD and potentially in venous thrombosis of unknown aetiology. Whilst externalisation of PE and PS was not altered in either disease state, a reduction in the procoagulant 12-HETE-PC was observed in UBD patients suggesting that there may be a defect in the pathway of how these phospholipids are synthesised and remodelled. Increased generation of 12-HETE-PE was observed in the thrombotic cohort supporting evidence of their role in promoting thrombin generation in human disease. In patients with UBD, a reduced ability of platelets and microvesicles to support thrombin generation was observed, however the thrombin generation assay as currently configured could not be used for diagnostic purposes. The increased ability of platelets to support thrombin generation in the thrombotic cohort may represent procoagulant factors other than the PL membrane composition not specifically tested here. For the management of bleeding in UBD patients, DDAVP is effective and may exert its procoagulant action by increasing PS externalisation in platelet disorders and bleeding disorders of undetermined aetiology. Finally, this thesis initiates investigation of procoagulant phospholipids in disorders of haemostasis and raises further areas for future research.

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Appendix

Unclassified Bleeding Disorder proforma

Study number:

Age: Gender:

Details of Family history:

Bleeding symptom up until diagnosis	**ISTH BAT score**
Epistaxis	Details: Cautery □ Packing □ Antifibrinolytic □ Blood Tx □ Replacement therapy □ **……**
Bruising	Details: Consultation □ Replacement therapy □ **……**
Bleeding from minor wounds	Details: Consultation □ Surgical haemostasis □ Blood Tx □ Replacement therapy □ **…….**
Oral cavity	Details: Consultation □ Surgical haemostasis □ Blood Tx □ Replacement therapy □ **…….**
Gastrointestinal bleeding	Details: Consultation □ Surgical haemostasis □ Blood Tx □ Replacement therapy □ **…….**
Haematuria	Details: Consultation □ Surgical haemostasis □ Blood Tx □ Replacement therapy □ **…….**
Tooth extraction	Details: Resuturing or packing □ Surgical haemostasis □ Blood Tx □ Replacement therapy □ **…….**

Surgery	Details: Surgical haemostasis □ Blood Tx □ Replacement therapy □ **……**
Menorrhagia	Details: Antifibrinolytics □ Hormonal therapy □ Iron therapy □ Replacement therapy □ …….
Post-partum haemorrhage	Details: Antifibrinolytics □ Hormonal therapy □ Iron therapy □ Replacement therapy □ …….
Muscle haematoma	Details: Surgical haemostasis □ Blood Tx □ Replacement therapy □ **……**
Other bleeding	
	Sum:-

Details of previous haemostatic challenges:

Number:

Antifibrinolytic □

Desmopressin □

Platelets □

Fresh frozen plasma □

Blood transfusion □

Other:

Outcome:

FBC	
MCV	
Platelets	
Relevant blood film findings	
APTT/PT/fibrinogen	
VWF Ag/Ricof/CBA/FVIII	
Blood group	
Platelet aggregation	
Platelet nucleotides	
FIX (males)	
FXI	
FXIII	

Deep vein thrombosis proforma

Study number:

Age: Gender:

Height:

Weight:

Body mass index:

Left calf: Right calf:

Presenting complaint:

RISK FACTORS		
Surgery in 12/52 □	Trauma lower limb □	Medical inpatient 12/52 □
Malignancy □	Previous DVT/PE □	Family Hx DVT/PE □
Varicose veins □	Childbirth 12/52 □	Pregnant □
Travel > 4 hrs □	BMI > 30 □	Immobile >3/7 □
	Inherited thrombophilia □	Contraceptive pill/HRT □
	Specify……..	IVDU □

Past medical history:

Alcohol:

Smoking:

Drug	**Dose**

Clinical examination findings:

Investigations and Results					
Hb		Na		Albumin	
Platelets		K		Alk phos	
WBC		Urea		ALT	
APTT		Creatinine		Bilirubin	
PT		eGFR		Protein	
MCV		CRP		Globulin	
Ferritin		D-dimer		Calcium	

Chest X-Ray (All patients with idiopathic DVT):

Ultrasound result

Right/Left

Above knee (Distal):

Peroneal vein □

Tibial vein □

Gastrocnemius □

Soleal □

Above knee (Proximal):

Popliteal vein □

Common femoral vein □

Femoral vein □

Superficial femoral vein □

Ileo-femoral □

Management plan:

Basal concentrations of APL in UBD and DVT patients

Basal APL	Healthy controls ng/4 x 10 ^{7} platelets mean values (+/- SE)	UBD patients ng/4 x 10 ^{7} platelets mean values (+/-SE)	
DOPS	0.31 (0.05)	0.18 (0.05)	P=0.07
SOPS	2.45 (0.31)	2.50 (0.26)	P=0.72
SAPS	2.01 (0.31)	2.08 (0.41)	P=0.99
SOPE	0.95 (0.17)	0.77 (0.20)	P=0.39
OpAPE	1.99 (0.33)	1.99 (0.37)	P=0.98
PpAPE	5.52 (0.68)	4.04 (0.81)	P=0.20
SpAPE	11.46 (2.76)	9.95 (2.00)	P=0.76
SAPE	7.13 (0.99)	8.84 (1.36)	P=0.28

Basal APL	Healthy controls ng/4 x 10 ^{7} platelets mean values (+/- SE)	DVT patients ng/4 x 10 ^{7} platelets mean values (+/-SE)	
DOPS	0.31 (0.05)	0.36 (0.04)	P=0.39
SOPS	2.45 (0.31)	2.48 (0.25)	P=0.76
SAPS	2.01 (0.31)	2.04 (0.39)	P=0.89
SOPE	0.95 (0.17)	0.60 (0.09)	P=0.42
OpAPE	1.99 (0.33)	1.67 (0.23)	P=0.66
PpAPE	5.52 (0.68)	6.30 (0.47)	P=0.45
SpAPE	11.46 (2.76)	11.23 (2.33)	P=0.85
SAPE	7.13 (0.99)	4.55 (0.57)	P=0.08

Patient	Age/gender	Diagnosis	Family history	Bleeding score at diagnosis	Details of haemostatic challenges after diagnosis	Bleeding outcome
PL009	75 F	UBD	No	• Bleeding post dental extraction (3) • Post-surgical: (4) ➢ Tonsillectomy (blood Tx) ➢ Anterior vaginal wall repair • Bruising (1) **BS=8**	Dental extraction *(TXA)* 2 x Colonoscopy and biopsies *(DDAVP & TXA)* Colonic endoscopic mucosal resection *(DDAVP & TXA)*	No bleeding
PL010	82F	UBD	Yes	• Epistaxis (3) • Bleeding post dental extraction (sutures) (3) • Post-surgical: ➢ Hysterectomy (FFP & cryoprecipitate) (4) • PPH X 2 (FFP & cryoprecipitate) (3) • Menorrhagia (4) **BS=17**	3 x Dental extraction *(TXA)* 2 x Cataract surgery *(TXA)*	No bleeding
PL011	82 M	UBD	No	• Bleeding from minor wounds (1) • Post-surgical: ➢ Total knee replacement (blood Tx) (4) **BS =5**	Orthopaedic hand surgery *(DDAVP & TXA)*	No bleeding

PL012	66F	UBD	No	• Bleeding post dental extraction x 2 (sutures) (3) • Post-surgical: (4) ➢ Hysterectomy ➢ Tonsillectomy (blood Tx) • PPH X 1 (2) • Menorrhagia (4) **BS=13**	4 x Orthopaedic hand procedures *(TXA)* Laparoscopic ventral rectopexy *(DDAVP & TXA)*	No bleeding
PL013	67F	UBD	No	• Bleeding post dental extraction (3) • Menorrhagia (4) **BS= 7**	Laparoscopic cholecystectomy *(no cover)* Colonoscopy & biopsies *(TXA)*	Postop haematoma and bruising around port entry with no cover
PL014	58F	UBD	Yes	• Bleeding post dental extraction (2) • Post-surgical: (4) ➢ Caesarean section (blood Tx) • Menorrhagia (4) • Easy bruising (1) **BS=11**	Endometrial ablation and insertion of miraena coil *(DDAVP & TXA)*	No bleeding
PL015	76F	UBD	No	• Easy bruising (2) • Bleeding post dental extraction (3) • Post-surgical: (2) ➢ Lumpectomy ➢ Haemorrhoidectomy • Bleeding from minor wounds (1) • Menorrhagia (2)	3 x Dental extraction *(TXA)* Cystoscopy *(TXA)* Laparoscopic hernia repair *(TXA)*	No bleeding

				BS =10		
PL016	62F	UBD	Yes	• Bleeding post dental extraction (3) • Post-surgical: ➢ Hysterectomy (blood Tx) (4) • PPH X 1 (1) • Menorrhagia (4) **BS=12**	No procedures	
PL019	60F	UBD	Yes	• Bleeding post dental extraction (sutures) (3) • PPH X 1 (blood Tx) (3) • Easy bruising (1) • Menorrhagia (3) **BS=10**	2 x Septoplasty (DDAVP *& TXA)*	No bleeding
PL020	78F	UBD	No	• Epistaxis (3) • Post-surgical: (4) ➢ Tonsillectomy (blood Tx) • PPH (blood Tx) (3) • Menorrhagia (4) **BS=14**	Removal of a skin lesion *(TXA)*	No bleeding
PL021	56F	UBD	Yes	• Bleeding post dental extraction (3) • Post-surgical: (4) • Bladder repair (blood Tx) • Knee arthroscopy • Menorrhagia (4) **BS=11**	Endometrial ablation *(DDAVP & TXA)* 3 x Knee arthroscopy *(DDAVP & TXA)* Breast biopsy *(DDAVP & TXA)* Dental extraction *(DDAVP & TXA)*	No bleeding

PL022	31F	UBD	No	• Menorrhagia (2) • Bleeding post dental extraction x 2 (3) • PPH X 1 (3) • Easy bruising (1) **BS=9**	Surgical stripping of varicose veins *(DDAVP & TXA)* Endometrial ablation & insertion of mirena coil *(DDAVP & TXA)*	No bleeding
PL027	47F	UBD	Yes	• Easy bruising (2) • Menorrhagia (4) • Post-surgical:(2) ➢ Hysterectomy **BS=8**	No procedures	
PL029	66F	UBD	Yes	• Bleeding post dental extraction x 2 (sutures) (3) • Post- surgical: (4) ➢ Cholecystectomy (blood Tx) ➢ Tonsillectomy • Menorrhagia (2) **BS=9**	Sebaceous cyst x 3 *(TXA)* Dental extraction *(TXA)*	No bleeding
PL031	76F	UBD	Yes	• Epistaxis (cautery) (3) • Bleeding post dental extraction (2) • Post-surgical: (4) ➢ Hysterectomy (blood Tx) ➢ Orthopaedic surgery • PPH (blood Tx) (3) • Menorrhagia (4) **BS=16**	Corrective foot surgery *(TXA)* Orthopaedic revision surgery *(TXA & DDAVP)* Laparoscopic pelvic floor repair *(TXA & DDAVP)*	No bleeding

PL032	61F	UBD	No	• Post-surgical: (2) ➢ Tonsillectomy • Bleeding post dental extraction (2) • Menorrhagia (3) • PPH (4) • Easy bruising (1) **BS=12**	5 X Dental extraction *(TXA)*	No bleeding
PL033	68F	UBD	Yes	• Bleeding post dental extraction (sutures) (3) • Post-surgical: (2) ➢ Tonsillectomy ➢ Lumpectomy • Easy bruising (2) **BS = 7**	Parathyroidectomy *(TXA and DDAVP)*	No bleeding
PL034	45F	UBD	No	• Bleeding post dental extraction (2) • Post-surgical: (4) ➢ Caesarean section • Menorrhagia (2) • Easy bruising (2) **BS=10**	Lumbar puncture *(TXA & DDAVP)*	No bleeding
PL035	56F	UBD	No	• Bleeding post dental extraction (3) • Easy bruising (1) • Post-surgical: (2) ➢ Lumpectomy ➢ Tonsillectomy • Menorrhagia (4) **BS = 10**	Discectomy *(TXA & DDAVP)* Bunion removal *(TXA & DDAVP)* Nissan fundoplication & banding of haemorrhoids *(TXA & DDAVP)*	No bleeding

PL036	58M	UBD	No	• Post-surgical: (2) ➢ Endoscopic polypectomy • Easy bruising (3) **BS=5**	Intranasal ethmoidectomy *(DDAVP & TXA)* Nasal polypectomy x 3 *(DDAVP & TXA; platelets for 3rd procedure)*	Platelets for 3rd procedure (bleeding with DDAVP & TXA alone)
PL037	76F	Scott syndrome	Yes	• Bleeding post-dental extraction (3) • PPH (3) • Intraocular bleed (retinal haemorrhage related to retinal vein thrombosis) (4) **BS=10**	Acute vitreal haemorrhage *(TXA & platelets)* Colonoscopy & biopsies x 2 *(TXA & platelets)*	No bleeding
PL039	53M	Abnormal PCI	Yes	• Muscle haematoma (3) • Ruptured ACL – haemarthrosis (3) **BS=6**	Dental extraction x 4 *(TXA)* Orthopaedic repair wrist *(TXA)* Arthroscopy *(Platelets & FFP)*	Oozing following 1 dental extraction (4 teeth removed), needed sutures
PL060	54F	PFD	No	• Easy bruising (2) • Minor wounds (1) **BS=3**	3 x Dental extraction *(TXA)*	No bleeding
PL061	67F	UBD	No	• Easy bruising (2) • Epistaxis (3) **BS=5**	Shoulder arthroscopy x 3 *(DDAVP & TXA)* Bunion removal (*DDAVP &TXA*)	No bleeding

F=Female; M=Male; UBD=Unclassified bleeding disorder; PCI=Prothrombin consumption index; PFD=Platelet function defect; BS=Bleeding score; DDAVP=Desmopressin; TXA=Tranexamic acid; Tx=Transfusion

Demographics of UBD patients and details of previous haemostatic challenges