

Measurement of Extracellular Vesicles by a Novel Fluorescent Imaging Method

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Abstract

Extracellular vesicles (EV) are small phospholipid delimited particles released by virtually all cells that cannot replicate. EV are difficult to measure, partially due to their small size and isolation contaminants including lipoproteins. EV isolation and measurement are not standardised and can be associated with bias in measured populations. An instrument that can measure EV in complex biofluids without the need for isolation may improve standardisation of EV research. The use of immunoaffinity capture combined with fluorescent labelling would allow for a greater depth of phenotypic characterisation and could have use diagnostically with EV increasingly being identified as biomarkers of health and disease. Here I test whether ExoView, a new chip-based immunocapture platform for EV measurement, can measure EV without the need for purification.

I generated platelet enhanced plasma (PEP) a platelet EV (PEV) enriched plasma with high procoagulant activity, as measured through thrombin generation and clotting assays. I designed a protocol for measurement of phosphatidylserine (PS) using ExoView which confirmed high PS expression on EV in PEP compared to platelet free plasma (PFP). I also investigate whether PEP may hold potential as a novel resuscitation fluid for use in austere environments where platelets are often unavailable.

Having found ExoView to be capable of measuring PS on EV, I next moved to a trace protein that is also involved in clotting: tissue factor (TF). TF is a lowly expressed protein that can be EV associated. In addition to historic EV measurement problems, TF has its own historic and ongoing controversies. However, a reliable method of TF EV quantification would be useful clinically in ovarian cancer, pancreatic cancer and venous thromboembolism. I utilised custom capture with ExoView to enrich TF

expressing EV from plasma followed by fluorescent labelling with a separate anti-TF clone and found ExoView can distinguish unstimulated vs lipopolysaccharide stimulated PFP. However, my TF EV method could not distinguish healthy PFP from clinical cohorts (cancer or trauma).

PEV and megakaryocyte (MK) derived EV are thought to be the largest EV population in circulation, however due to their similar parental lineage unique markers of either are difficult to identify and validate. They are interesting to distinguish as a change in the ratio of PEV:MK EV may indicate an increased risk of cardiovascular disease or may reflect bleeding risk in patients on antithrombotic therapeutics. I utilised ExoView and theoretically unique markers of PEV and MK EV on purified platelet supernatants and MK cell culture supernatants to try and identify unique signatures of PEV and MK EV which were then applied to PFP. The set of markers I used and the conditions I tested did not reveal a unique signature of PEV or MK EV that could be used to determine whether PEV or MK EV are more the more dominant EV source in circulation.

In conclusion, this thesis shows that ExoView has great capacity for EV measurement both in basic characterisation of EV-enriched markers such as tetraspanins, but also has potential for measurement of clinically relevant, rare subpopulations of EV.

Publications

Publications arising from this thesis

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UKEV 2023: Comprehensive functional characterisation of a novel ANO6 variant in a new patient with Scott Syndrome.

UKEV 2023: The role of adipose-derived extracellular vesicles in mediating the obesity associated decline in musculoskeletal health with ageing.

List of Abbreviations

AFC	Automated fraction collector
AFM	Atomic force microscopy
APTT	Activated partial thromboplastin time
BD	Becton Dickinson
BSA	Bovine serum albumin
Ca ²⁺	Calcium
CASCADE	Cardiovascular and bleeding outcomes associated with antithrombotic therapy
CAT	Calibrated automated thrombography
CFT	Clot formation time
COVID-19	Coronavirus disease 2019
CRP	Collagen-related peptide
CSF	Cerebral spinal fluid
CT	Clotting time
CVD	Cardiovascular disease
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
dUC	Differential ultracentrifugation
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
EMT	Epithelial-mesenchymal transition
eNOS	Endothelial nitric oxide synthase
ESCRT	Endosomal sorting complex transport
ETP	Endogenous thrombin potential
EV	Extracellular vesicles
FBS	Foetal bovine serum
FFP	Fresh frozen plasma
GPRP	Gly-Pro-Arg-Pro
HBSA	Hepes buffer saline with bovine serum albumin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPL	Human platelet lysates
ILV	Intraluminal vesicles
IPM	Infusible platelet membranes
IPSC	Induced pluripotent stem cell
ISTH	The International Society for Thrombosis and Haemostasis
KO	Knockout
LAMP-1	Lysosome-associated membrane protein-1
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
m/IEV	Medium/large extracellular vesicle
MCF	Maximum clot formation
miRNA	Micro-RNA
MK	Megakaryocyte
MSCT	Mesenchymal stem cell therapy
MSCT	Mesenchymal stem cell
MV	Microvesicles
MVB	Multivesicular bodies
NET	Neutrophil extracellular trap
NHSBT	National health service blood and transport
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
NTA	Nanoparticle tracking analysis
OA	Osteoarthritis
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PEP	Platelet enhanced plasma
PEV	Platelet extracellular vesicle
PFP	Platelet free plasma
PPP	Platelet poor plasma
PRP	Platelet rich plasma

PS	Phosphatidylserine
PT	Prothrombin time
RNA	Ribonucleic acid
ROTEM	Rotational thromboelastometry
SEC	Size-exclusion chromatography
SEM	Scanning electron microscopy
sEV	Small extracellular vesicle
SNAREs	Sensitive factor attachment protein receptors
SP-IRIS	Single particle interferometric reflectance imaging sensor
SSC	Scientific and standardisation committee
TEM	Transmission electron microscopy
TEMS	Tetraspanin enriched microdomains
TF	Tissue factor
TRPS	Tuneable resistive pulse sensing
VLDL	Very low density lipoprotein
VTE	Venous thromboembolism
vWF	von-Willebrand factor

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COVID-19 Mitigation Statement

The COVID-19 pandemic significantly impacted the research originally planned for this thesis. During the COVID-19 pandemic I had limited access to the laboratory and to blood donors. This thesis focuses on circulating EV and therefore relies heavily on access to blood donors. In addition, COVID-19 impacted on access to training, limited allowed working hours, and led to self-isolation periods that all contributed to delayed research required for this thesis. The single largest change due to COVID was the absence of cardiovascular disease patients due the CASCADE clinical trial closing at an early stage of recruitment. This necessitated refocussing this thesis to existing clinical cohorts with banked samples and altering experimental plans.

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Chapter 1. Introduction

1.1 Extracellular Vesicles

Extracellular vesicles (EV) are released by all cells and have been identified in virtually all biological fluids including blood, urine, serum, milk and cerebrospinal fluid (CSF) [1]. They are small cell-derived particles that cannot replicate and are delimited by a lipid bilayer [2, 3]. Modern classification divides EV into 2 major sub-types i.e. those of endosomal (i.e. exosomes) or plasma membrane origin (i.e. microvesicles and apoptotic bodies) as shown in Figure 1.1.

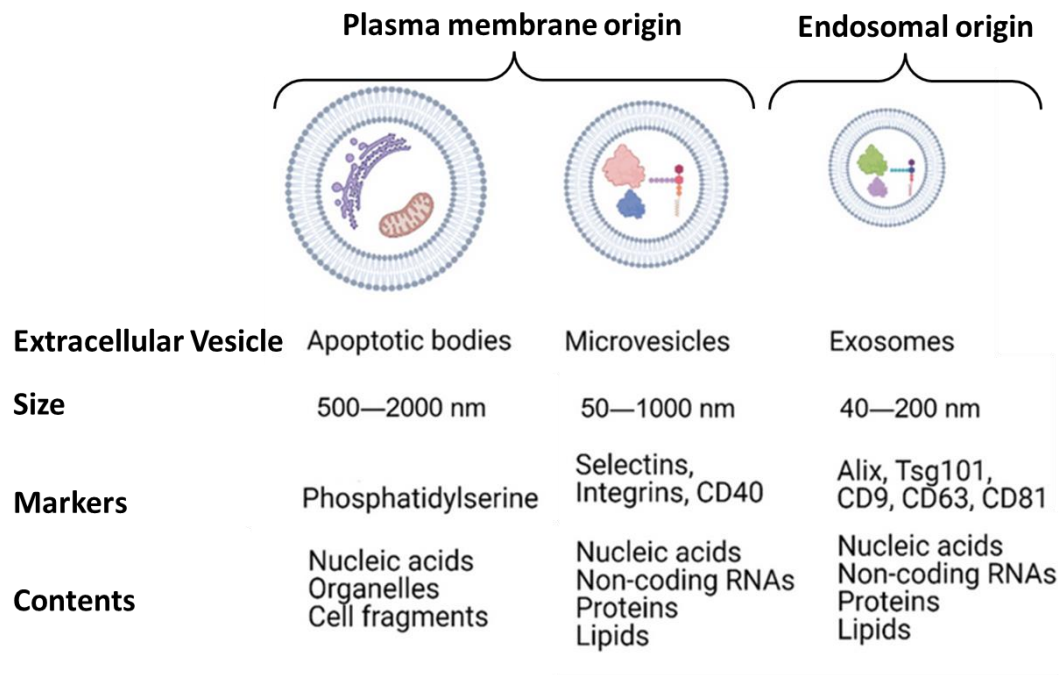


Figure 1.1. The classification of EV based upon their origins and properties.

EV are broadly split by their cellular origins from either the plasma membrane (apoptotic bodies and microvesicles) or the endosome (exosomes). Their size distributions, commonly used markers and cargo are shown.

To understand arrival at modern nomenclature it is necessary to delve into the history of EV. In 1967, Wolf described sedimented material or “platelet dust” derived from platelet poor plasma (PPP) with platelet-like procoagulant properties and generated electron microscopy (EM) images of the material [4-6]. Platelet dust was the first observation of what are now termed EV [6]. Removal of platelet dust by ultracentrifugation reproducibly increased the clotting time of PPP thereby showing functional significance thus confirming the earlier observations by Chargaff in 1946 [4]. By 1971, Crawford had shown that this material, then referred to as microparticles, contained lipid and carried protein [7]. Other investigators found similar microparticle material released from alveolar cells [8]. In the 1960s “matrix vesicles” were identified, imaged and shown to participate in bone mineralisation [9, 10]. A key finding in the 1970s was the presence of multivesicular bodies close to the apical membrane of particle releasing cells [11]. Also, in the late 1960s and early 1970s virologists were exploring a viral link to cancer and repeatedly found non-viral particle material in both healthy controls and patient plasma samples [12-18].

However, it wasn't until the 1980's that the term exosome was also suggested to describe membrane shed particles [19]. Exosomes were first identified to be important for red cell reticulocyte maturation by mediating the release of the transferrin receptor, not by membrane shedding, but through multivesicular body mediated exocytosis [20, 21]. Rose Johnstone who played a key role in this reticulocyte research is often quoted as stating that “EV are a major route for externalisation of obsolete membrane proteins” [22]. This sentiment of EV as primarily waste disposal has remained for decades [23]. However, over those decades much understanding has also been

gained on EV biogenesis and function. EV have been recognised as key mediators of intercellular communication with roles in homeostasis and disease [24]. They can be targeted to distal sites and can mediate transmission of signals essential for homeostasis and health; for the propagation and progression of disease states and also promoted as therapeutics to treat disease [24]. EV are widely conserved across the animal and plant kingdoms and are now recognised as an ancient communication system between bacteria [25]. Growing evidence suggested that bacterial EV could interact with mammalian and plant cells [25, 26]. Cardiovascular disease has been repeatedly linked to higher concentrations of circulating EV particularly those of platelet origin [27-30]. Immune cell EV were shown to be capable of antigen presentation in 1996 [31]. This was an important milestone discovery that ignited interest in utilising custom EV to generate specific vaccines, for example against tumour specific protein [32, 33].

Unfortunately, the growing EV field came with a cacophony of overlapping terms to describe vesicles [23]. Nomenclature included exosome, microvesicle, microparticle, ectosome, virus-like particles and equenas particulas [23]. Not only was this confusing but these differing categories were often arbitrarily chosen by authors or were purely based on the size of the particle studied [34]. However, it is now appreciated that EV are highly heterogeneous, varying and overlapping in their size, cargo (which can include various ribonucleic acid (RNA) species, deoxyribonucleic acid (DNA) species, protein and lipid), and biological functions [35-37]. These features alone, therefore, cannot always determine whether EV are endosomal or plasma membrane derived [2]. In the early 2010's a growing number of publications and meetings were calling for

more specific nomenclature and the term EV was suggested as a universal term for extracellular lipid bilayer delimited vesicles that cannot replicate [38]. Although exosomes and MV were thought to be EV of exclusive endosomal or plasma membrane origins respectively, the term exosome was also perceived as a more desirable or popular term by many cell biologists and EV researchers especially those involved in therapeutic applications of EV [34].

Nomenclature was recognised as being important for an emerging field and the suggestion of EV as a collective term was formalised in 2012 with the foundation of the International Society of Extracellular Vesicles (ISEV) and the Journal of Extracellular Vesicles (JEV). Researchers from all of the emerging vesicle fields unified and began to recognise the common problems and important issues with EV research. These were summarized and continuously updated in a pioneering series of position papers in JEV entitled “Minimal information for studies of extracellular vesicles” (MISEV2014, 2018 and 2024 (in press)). These established current nomenclature recommendations but also emphasised the difficulty of EV categorisation without use of live imaging techniques or direct measurement of validated specific markers of subcellular origin [2]. Instead MISEV recommends EV categorisation to be based on physical, measurable characteristics of EV including size, density, biochemical composition and cellular origin [2]. This recommendation has led to a change in reporting EV research, with the terms exosome and microvesicles reserved for use when either endosomal or plasma membrane origin can be definitively proven [2]. Nomenclature today largely consists of small EV (sEV, < 200nm), medium EV/large EV (m/lEV, > 200nm), reporting of marker expression,

density, and/or cellular origin [2]. EV research is a growing and emerging field where reproducibility of results is important and standardization and nomenclature are now an important foundation for modern EV research. However, many problems and issues still remain, including improving and standardizing both EV isolation and measurement techniques. A key focus of this thesis is establishing ExoView as a novel platform for EV measurement.

1.1.1 Exosomes

Exosomes (<100 nm) originate from the endosome and can be secreted either constitutively or by cellular activation [39]. An initial inward budding of the cell membrane forms early endosomes, which develop into late endosomes by undergoing further inward budding of the endosomal membrane [40]. A population of late endosomes, multivesicular bodies (MVB), contain intraluminal vesicles (ILVs). A proportion of MVB undergo lysosomal degradation [40]. Some MVB enter the endocytic pathway, appropriate plasma membrane surface proteins and undergo endoplasmic reticulum processing and golgi complex cargo loading [40]. Despite the common endosomal derivation, exosomes are highly heterogeneous and multiple overlapping pathways are responsible for their formation. ILV formation can be regulated by the endosomal sorting complex transport (ESCRT) [41]. ESCRT is composed of four well-conserved complexes and a host of associated proteins (VPS4, VTA1, ALIX, CHMP4C) [42]. Understanding of ESCRT-dependent MVB formation is well established from studies on lysosomal degradation [39]. Proteomics has confirmed the presence of ESCRT components enriched in EV, with TSG101 often implicated. Knockout (KO) and RNAi silencing studies have shown that different

ESCRT components influence the composition and number of EV generated by various cells [41, 42]. Equally, inhibition of ESCRT-III accessory proteins such as ALIX have been shown to increase exosome release in some cell types and reduce exosome release in others [43, 44]. Another key pathway under ESCRT mediated EV biogenesis is the syndecan/syntenin/ALIX pathway. Syntenin is a scaffold protein that interacts with a wide range of signalling pathways, including Wnt or integrin signalling and through interaction with TGF- β can promote epithelial–mesenchymal transition (EMT), invasion, and metastasis [44, 45]. There is redundancy in proteins involved in EV biogenesis and release as well as cell-specific phenomena, whereby the loss of single proteins can alter exosome production [44].

ESCRT-independent processes operate separately and in tandem with ESCRT-dependent processes in the formation of exosomes. ILV formation has been shown to occur even when all four ESCRT-complexes are simultaneously silenced [46]. ESCRT-independent exosome biogenesis is less well-characterised but is contributed to by lipid raft organisation, CD63 dependent mechanisms and CD81-mediated cargo selection [42]. ESCRT-independent exosome biogenesis has also been shown to occur whereby local lipid composition coupled with a pH gradient is sufficient to form ILVs [47]. ILVs are released from MVB upon fusion with the plasma membrane and are subsequently termed exosomes. As with biogenesis, secretion of exosomes involves multiple mechanisms, with several levels of regulatory control that can vary in a cell dependent manner [42]. Although there are multiple ESCRT independent exosome biogenesis pathways, one well established and important mechanism is linked to tetraspanins. Tetraspanins represent a superfamily of proteins responsible

for organising membrane microdomains through clustering and include the classic exosomal markers CD63, CD9 and CD81 [48]. Tetraspanin enriched microdomains (TEMS) serve as cargo transportation platforms and are directly involved in EV cargo loading, cargo selection and have been proposed as specific EV markers [49, 50]. Knockout of CD9 for example has been shown to reduce exosome production in dendritic cells [51]. Interestingly, although involved in ESCRT independent biogenesis CD63 can also bind syntenin and is also involved in ESCRT mediated biogenesis [52]. The conventional thinking is that tetraspanins are recruited in both ESCRT and ESCRT-independent mechanisms and undergo endosomal endocytosis and therefore aid in passage of MVB to the plasma membrane and their subsequent release[48, 50]. Tetraspanins and syntenin have therefore been used as candidate specific markers for exosomal origin. Figure 1.2 shows the conventional model of EV biogenesis.

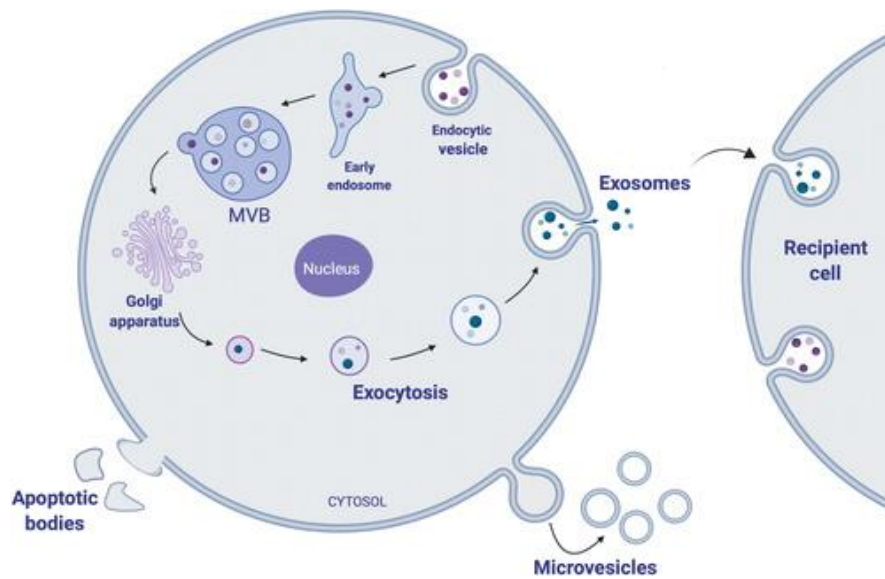


Figure 1.2. Biogenesis pathways for EVs.

Exosomes are derived from multi-vesicular bodies and are released by exocytosis, whereas microvesicles are generated by plasma membrane blebbing. Apoptotic bodies tend to be larger and are released from apoptotic cells. Figure modified from [53].

A recent model (Figure 1.3) suggests an overhaul of current ESCRT vs non-ESCRT processes and challenges the exclusive endosomal origin of exosomes, instead promoting the idea that all EV biogenesis is primarily plasma membrane driven, with two major pathways: 1, direct shedding and 2, endocytosis and endosome involvement. This model suggest that the tetraspanins at the plasma membrane are responsible for exosome release and their endosomal endocytosis leads to reduced exosome output [54]. They suggest that syntenin's primary role is to inhibit CD63 endocytosis and drive the plasma membrane accumulation of CD63, with exosome production and release from the plasma membrane [54]. However, Fan *et al* suggest that tetraspanins have a very limited role in determining the protein composition of exosomes [55]. However, they only used CD81 KO to determine this and as previous studies have shown there is redundancy in exosome biogenesis pathways that may be overcome via expression of other tetraspanins [55].

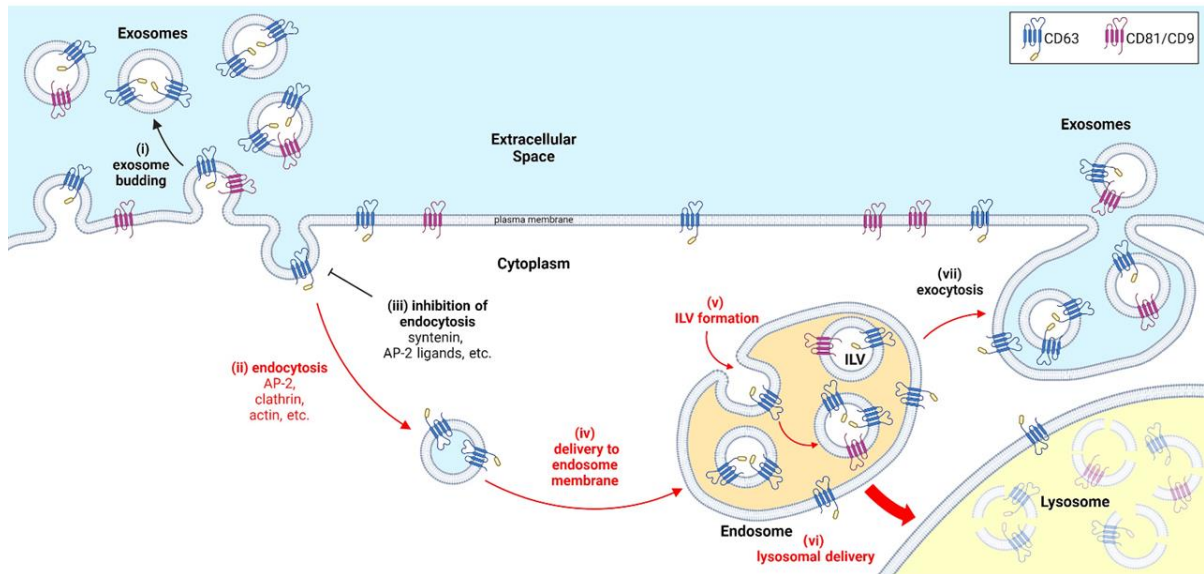


Figure 1.3. A new model of EV biogenesis.

Developed by Ai *et al* this model of EV biogenesis does not distinguish between exosomes and microvesicles and suggests that blebbing from the plasma membrane is the primary route of exosome biogenesis, and lysosome/multivesicular body derived EV are an alternative route for exosome biogenesis. Adapted from [54].

For ILV release to occur MVBs must not be signalled for lysosomal degradation, which can be initiated by induction of ISGylation [56]. The mechanism by which the fate of MVBs is determined is unknown but is influenced by cellular stress [57-59]. Cells under stress tend to increase exosome secretion and this may be caused by the increased workload of lysosomes or to enable communication with neighbouring cells. For example the use of ammonium chloride to induce lysosome dysfunction promotes increased EV release [60]. It is feasible that in disease, especially aging associated diseases where autophagy is impaired the EV secretome may therefore be altered.

To reach the plasma membrane MVBs interact with actin and the microtubule cytoskeleton with a key role for the actin binding protein cortactin as documented by live-cell imaging [61]. Cortactin overexpression also leads to increase EV release [61]. RAB GTPases have also been implicated in exosome secretion, though vary between species and between cell types within the same species which suggest redundancy in their individual actions [62]. Alternatively, this may facilitate a system for fine-tuning of EV secretion dynamics.

Upon reaching the plasma membrane, in order to be released, MVB must overcome the energy requirements for fusion and subsequent exosome release [63]. Both protein-protein and protein-lipid interactions aid in reducing the energy requirement for fusion. Although the mechanism for fusion is not clearly understood, it likely involves sensitive factor attachment protein receptors (SNAREs) and may be promoted by increases in intracellular calcium concentration. Tetraspanins also play a role in plasma membrane fusion, both in exosome release and their uptake by recipient cells. In MVB fusion with the plasma membrane CD81 promotes the outward curvature of

the plasma membrane [64]. Some of the tetraspanin effects on plasma membrane curvature are mediated by altering the local lipid composition of the membrane, or through indirect interaction with the cytoskeleton via GTPases and interaction with ezrin, radixin and moesin [64].

1.1.2 Microvesicles

Microvesicles (MV) (100-1000 nm) are generated through outward blebbing of the plasma membrane [65]. MV are released from cells under physiological conditions, particularly in response to cellular activation with and without injury, inflammation and stress (oxidative, shear) [2]. During the blebbing process negatively charged phospholipids are translocated from the inner plasma membrane to the outer membrane of the released MV [66]. Therefore, phosphatidylserine (PS) is often used as an MV marker [66]. The study of MV has advanced largely through their involvement in the pathophysiology of cancer and cardiovascular diseases [67-78]. However, it is worth noting that authors have used multiple naming systems for MV, including ectosome, shedding vesicle and microparticle. In many cases, MV may be referring to exosomes and so searching the literature for specific MV function can often be challenging [2]. In cancer MV, have been linked to metastasis [79-81]. Transformation of cells *in vitro* has also been shown to increase MV production and release [82]. MV biogenesis involves organised restructuring of the plasma membrane, including phospholipid redistribution, actomyosin contractile machinery and protein composition changes. Lipid and membrane-associated proteins cluster in the plasma membrane and recruit RNA, DNA, lipid and/or protein cargo for MV packaging. These clustered microdomains also recruit machinery that enable localised

sites of membrane asymmetry to be altered. Membrane asymmetry of cells is normally characterised by higher amounts of phosphatidylcholine and sphingolipids in the extracellular leaflet and greater amounts of phosphatidylinositol, phosphoinositide and phosphatidylserine in the cytosolic leaflet [83]. The asymmetry of the plasma membrane is maintained by the action of flippases, which catalyse movement of phospholipid species from the extracellular leaflet to the cytosolic leaflet [84]. Floppases mediate phospholipid movement in the reverse direction and are important in MV budding. Scramblases are ATP-independent and are capable of bidirectional movement of phospholipids, this is termed “flip-flop” activity [85]. Scramblases and floppases are calcium sensitive, their activity alters local membrane structure and causes changes in actin dynamics which leads to physical bending of the membrane and promotes MV formation [86, 87]. However, MV release is largely contingent upon an increase in cytosolic calcium concentration and consequently calpains are activated promoting cytoskeletal modification and remodelling [14-16]. Increased cytosolic calcium concentration inhibits flippase activity and increases floppase and scramblase activity, this disrupts membrane asymmetry and mediates externalisation of PS and other negatively charged phospholipids [86, 87]. In some cells this activity is influenced by ATP-driven activation of P2X7 receptors. P2X7 activation, leads to the phosphorylation of p38 and subsequent activation of acid sphingomyelinase which converts sphingomyelin to ceramide [88]. Ceramide is a cone-shaped lipid that alters membrane curvature [88]. Together membrane asymmetry and cytoskeletal modifications mediate outward blebbing from the plasma membrane and MV release [65].

1.1.3 Apoptotic bodies

Apoptotic bodies (1-5 μm) are formed by blebbing from the plasma membrane of cells undergoing apoptosis [89]. Apoptotic bodies, cover the greatest size range of EV and are more likely to contain cell organelles and nucleic acids (e.g. DNA) [89]. As with MV, membrane asymmetry is disrupted and PS is exposed, acting as an “eat me” signal to macrophages to mediate clearance [89]. See Figure 1.2 for an overview of EV biogenesis.

1.1.4 EV uptake in recipient cells

EV have been shown to be taken up by cells via direct fusion, phagocytosis and endocytosis through overlapping mechanisms including caveolin-mediated, lipid-raft mediated, clathrin-dependent and clathrin- independent processes [90]. EV adhere to target cells through a combination of tetraspanins [91], integrins [92] and proteoglycans [93]. EV uptake is rapid *in vitro* with EV accumulation occurring within 15 minutes and reduced accumulation occurring at 4°C suggesting energy dependent pathways are involved [94]. Furthermore, inhibitors such as Cytochalasin D substantially reduce EV uptake, implicating cytoskeletal components such as actin [95]. The failure to completely block EV uptake when actin is depolymerised suggests other mechanisms are also involved in EV uptake. Clathrin coating of EV can occur at the plasma membrane in regions called clathrin pits where they promote cell membrane deformation, inward budding and eventual pinching of EV into the cell. This process is independent of actin and reliant on GTPases such as Dynamin2 [96]. As well as clathrin mediated EV entry, there are regions of the plasma membrane that form small invaginations called caveolae. These regions are rich in caveolins,

cholesterol and sphingolipids and form glycolipid rafts that locally alter plasma membrane dynamics and promote curvature enabling endocytic events [97]. Lipid rafts at localised regions altered phospholipid composition and are thought to be involved across multiple endocytic pathways [98]. It is difficult to conclude whether EV release is directed with specific target cells *in vivo*, as *in vitro* data suggests EV from multiple sources are taken up by virtually all cell types tested, although some groups report specific enrichment in target cells [90]. However, Andaloussi's research group suggest engineering EV with specific targeting motifs is the most reliable way to target EV to specific cells [99]. An additional interesting point is that clathrin and caveolin mediated endocytosis ultimately lead to the endosome where the primary fate of EV and their cargo is degradation [100]. This has important implications for the relevance of *in vivo* cell-cell EV mediated communication and is an area where EV engineering science is currently focused.

1.2 EV as biomarkers and therapeutics

EV are strong candidate diagnostic and prognostic biomarkers and may hold novel therapeutic potential. Increasingly, evidence suggests that the benefits of mesenchymal stem cell therapy (MSCT) are mediated by mesenchymal stem cell (MSC)-derived EV [101, 102]. Preclinical studies of EV from a diverse range of sources show therapeutic potential of EV in various disease states and conditions. In addition to the therapeutic potential of EV, they can also promote disease progression and therefore form novel drug targets. For example, neutrophil-derived EV can carry

neutrophil elastase in a form that blocks alpha1-anti-trypsin activity and subsequently promote chronic obstructive pulmonary disease progression [12].

1.2.1 Biomarkers

Blood biopsy has gained significant interest as a source of EV-associated biomarkers due to the relative ease and accessibility of this sample type in the clinical setting [103]. This is now a key area that has yielded results is in cancer. Glypican-1 is a cell surface proteoglycan that has been found enriched on EV in serum from pancreatic cancer patients where it correlates with tumour burden and survival in patients [104]. Serum exosome-associated miR-373 can differentiate between breast cancer subtypes [105]. Micro-RNA (miRNA) and long-noncoding RNA signatures identified in circulating EV have been shown to match signatures from tumour sites in non-small cell lung cancer patients [106]. This highlights the interesting possibility of monitoring circulating EV to determine whether remission is stable.

Another easily accessible biofluid is urine, which has been shown to harbour EV with predictive value following kidney transplantation, with the presence of neutrophil gelatinase-associated lipocalin cargo in EV a better predictor of kidney dysfunction than other urinary fractions [107]. Saliva is easily accessible and saliva EV have been proposed as biomarkers for pancreatic cancer [108], and lung cancer [109]. Furthermore, miRNA signatures in EV are being investigated in the saliva and urine of professional rugby players to identify an EV associated signature of concussion, if successful this could lead to the development of a technology to identify players at risk of concussion as a pitch-side test and remove them from play [110]. Cerebral spinal fluid (CSF) EV from Alzheimer's patients have been shown to carry significantly

increased levels of total tau and phosphorylated tau [111]. Moreover, two Parkinson's disease-related proteins, LRRK2 and DJ-1, have been identified in urinary and CSF exosomes [112].

1.2.2 Therapeutics

MSCT is a therapeutic technique that has been used to treat degenerative diseases affecting the cardiovascular system and the musculoskeletal system [113]. MSCT delivery is often via intravenous infusion [114, 115]. It has been shown that a large proportion of MSC become trapped in capillary beds and the lungs, thus potentially reducing their therapeutic effect [115]. Despite this there is good evidence some positive effects are imparted upon degenerative tissue. This suggests that some MSC still reach their target tissue. An alternative explanation is that MSC can release EV that also mediate the therapeutic effect. Interestingly, MSC EV have been shown to have similar effects as MSC in mouse models of osteoarthritis (OA) [116]. Some emerging evidence suggests that EV derived from mesenchymal stem cells that highly express CD10 have beneficial effects on cartilage in OA and may directly interact with substance P to reduce patient pain [101].

EV also lend themselves to bioengineering. EV can be generated with specific cargo and homing markers to deliver small molecule therapeutics to desired sites. Engineering EV is an ongoing approach with multiple different avenues. For example a coronavirus disease 2019 (COVID-19) vaccine has been produced based on engineering EV that express viral antigens and are targeted to T cells to generate an effective immune response [117, 118]. Another strategy involves constructing artificial EV by integrating cell membrane proteins and lipids in the presence of target

molecules (drugs, surface makers to target specific cells, etc.) [119]. A key problem with EV-based therapeutics is standardising the formulation used as it must be rigorous and reproducible. This is difficult with bioreactors where EV concentrations and compositions may alter from passage to passage. There is some evidence that the presence of foetal bovine serum (FBS) (present in MSC culture) can cause an immune response in recipients [120]. However, Bobis-Wozowicz *et al* show that xeno-free conditions can produce MSC EV with similar therapeutic effects *in vitro* [121]. Andaloussi *et al* have designed intricate systems that utilise a mixture of viral proteins and crispr-CAS9 technology to create custom EV with specific targeting motifs and EV with increased half-life. They have also utilised unique cargo release technology to reduce endosomal degradation of EV cargo upon cellular entry [122].

1.3 Platelets, tissue factor and megakaryocyte EV

1.3.1 Platelets

Platelets are anucleate cells produced by their parent cell, the megakaryocyte which are in turn derived from common myeloid progenitor cells derived from pluripotent hematopoietic stem cells [123]. As anucleate cells, platelets have limited ability to *de novo* synthesise new proteins but do contain mRNA transcripts and translation machinery [124, 125]. Platelets are approximately 2-3 μm in diameter with a lifespan of up to 10 days in circulation [123]. Platelets form a central and important role in all phases of haemostasis (primary haemostasis, secondary haemostasis, and fibrinolysis) [126]. Under resting conditions endothelial cells suppress adhesion of platelets and prevent thrombus formation. However, upon vessel wall damage various proteins including von-Willebrand factor (vWF) and collagen are released or exposed.

Platelets bind VWF via GPIb-IX-V. vWf-GPIb-IX-V interactions are unstable resulting in rolling and translocation across the injury site but are stabilised through $\alpha_2\beta_1$ collagen interactions and collagen mediated GPVI signalling leads to the activation of platelet integrin CD41/CD61 ($\alpha_{IIb}\beta_3$) [127, 128]. $\alpha_{IIb}\beta_3$ binds fibrinogen, forming inter-platelet connections to promote aggregation leading to thrombus formation [129]. Throughout this process platelet activation is occurring, leading to shape change (pseudopod formation), platelet spreading and granule release to further promote platelet recruitment. The plasma membrane of activated platelets is also reorganised, losing lipid asymmetry and increasing exposure of negatively charged phospholipids such as PS [130, 131]. Platelet derived dense (δ) granules release large amounts of ADP which act on P2Y1 and P2Y12 platelet receptors to further activate platelets [132]. Fibrinogen and VWF are secreted from platelet alpha (α) granules and continue to propagate aggregation events [133]. Alpha granules also release P-selectin (CD62P) which recruits leucocytes including monocytes and neutrophils [133]. The recruitment of immune cells promote the initiation of the inflammatory response [134]. As platelets are recruited and spread they form a thrombus over the endothelial injury and thereby block bleeding [123].

1.3.2 Clotting Cascade

Platelet activation events interact with the clotting cascade bidirectionally [135] The clotting cascade can be subdivided into the extrinsic, intrinsic and common pathways (Figure 1.4). The clotting cascade acts as an amplification of small amounts of activating signals, ultimately promoting thrombin generation and cleavage of soluble fibrinogen to form insoluble fibrin. Fibrin crosslinks and interacts with platelets via $\alpha_{IIb}\beta_3$

to stabilize the clot. The extrinsic pathway is largely initiated by tissue factor (TF/CD142), a transmembrane protein essential for normal haemostasis. TF is expressed by vascular smooth muscle cells, pericytes and adventitial fibroblasts within and surrounding the blood vessel wall [136]. Traditionally, it was thought that, upon injury, the exposure of TF in an extra-vascular manner promotes haemostasis. This view has now been modified in consideration of circulating forms of TF [137, 138] (see section 1.3.5 Tissue Factor and EV).

TF binds FVII and FVIIa; the TF-FVIIa complex activates FIX and FXa, acting as a spark for the rest of the coagulation pathways. The extrinsic pathway is clinically measured as the prothrombin time (PT). The intrinsic pathway begins with FXII which is activated by contact activation. Once activated FXIIa activates FXI, which in turn activates FIX where the common pathway of FX to FXa conversion is then reached. The intrinsic pathway is clinically measured as the activated partial thromboplastin time (APTT). Conversion of X to Xa is mediated by the tenase complex, which exists in two active forms: 1) FVIIa:TF and Ca^{2+} ; 2) FVIIIa:FIXa:phospholipid and Ca^{2+} . Externalised PS on activated platelet membranes provides a negatively charged surface that promote assembly of the tenase and prothrombinase complexes, which activate FX and catalyse the cleavage of prothrombin to form thrombin [139]. Thrombin in turn activates platelet PAR1 and PAR4 receptors and acts to cleave fibrinogen to form fibrin [139]. Thrombin also activates clotting factors FXI, FV, FVIII and FXIII, thus forming a feedback loop. Additionally, thrombin provides negative feedback by converting plasminogen to plasmin and through production of antithrombin [140].

Plasmin acts to degrade the fibrin mesh and antithrombin reduces the production of thrombin from prothrombin [141].

The clotting cascade acts like a waterfall or an avalanche, the starting concentration of the initial factors of the intrinsic and extrinsic clotting cascade are low, but their activation triggers further amplifications that quickly cascade out to a rapid clotting response. Figure 1.4 shows a simplified illustration of the clotting cascade.

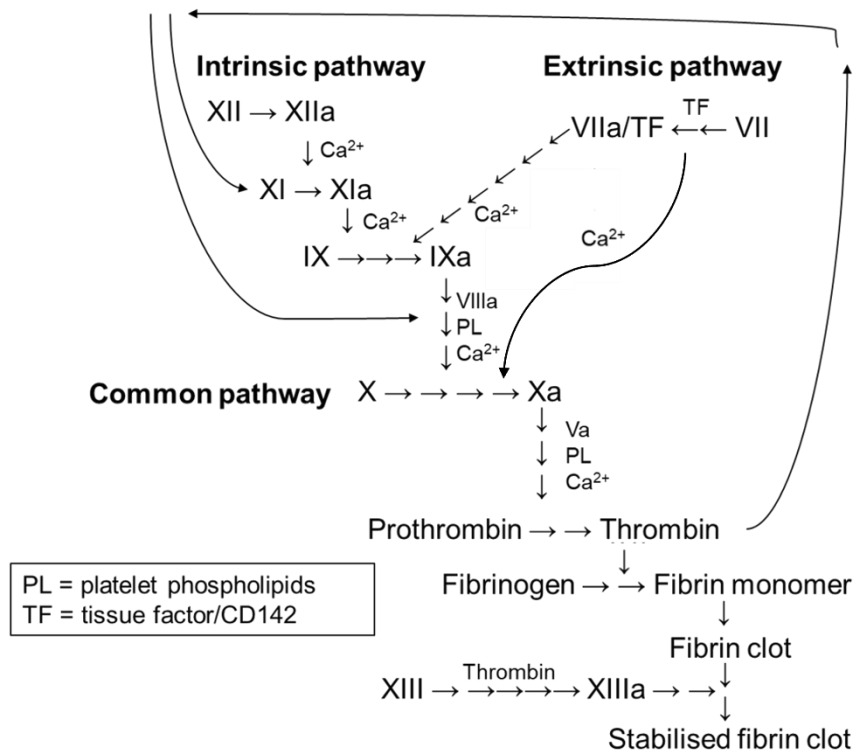


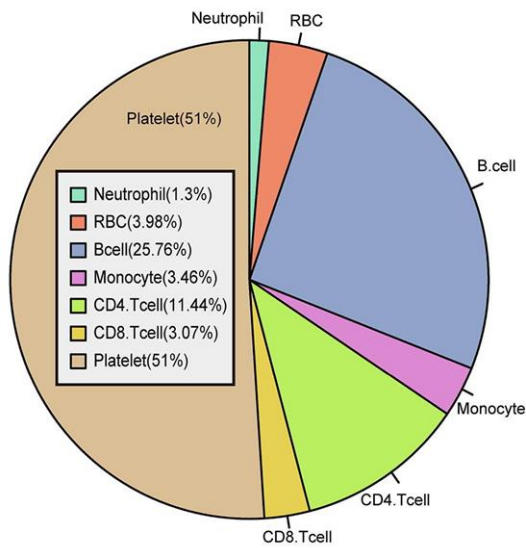
Figure 1.4. The clotting cascade.

The extrinsic, intrinsic and common pathway of the coagulation cascade. The extrinsic pathway is often referred to as a “spark” the VIIa/TF complex is responsible for initiating the cascade and leads to thrombin generation, activation of components of the intrinsic cascade and sustained activation of the common pathway. Ultimately these pathways overlap and converge and promote fibrin formation, polymerisation, crosslinking and the formation of a stabilised clot.

1.3.3 Circulating EV

EV are released into circulation from platelets, erythrocytes, leucocytes, cells of the endothelium and from tissue. EV have been found to cross the blood brain barrier, so both circulating EV can reach the brain and neuronal EV can reach the circulation. Li *et al* estimate that 51% of EV in circulation are from platelets, 48.8% from leucocytes and the remaining 0.2% of circulating EV originate from tissue, with 80% of that adipose derived (Figure 1.5) [142]. Li *et al* utilised long non-coding RNA signatures of tissue and of plasma derived EV to make their estimates, however the raw data they used to calculate this is not shared and the algorithm used has not been made public so has been difficult for other authors to reproduce. Nonetheless, others have repeatedly shown PEV or MK EV to be the dominant source of circulating EV [143, 144].

A



B

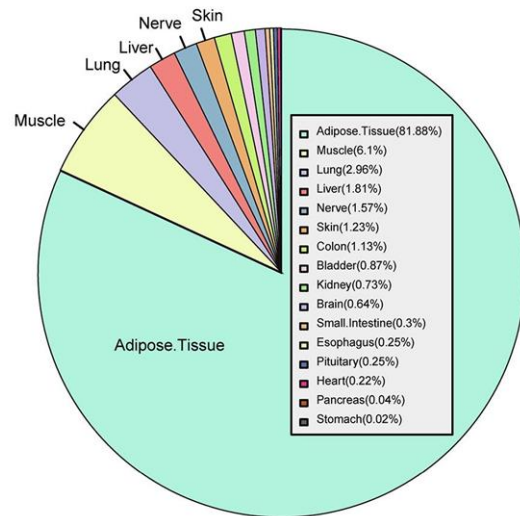


Figure 1.5. Cellular and tissue origin or EV in circulation.

a) The relative distribution of all blood cell derived EV (Totalling 98.8% of circulating EV). (b) The pie plot shows the relative comparison of the remaining 0.2% of circulating EV from and 16 types of tissue. Adapted from [142].

1.3.4 Platelet EV (PEV)

Platelets have roles beyond haemostasis through interactions with monocytes, macrophages, T cells, neutrophils, and natural killer cells [53– 57]. They can recruit immune cells through release of cytokines and complement factors [59], they form aggregates with neutrophils and stimulate neutrophil extracellular trap (NET) production [56,58]. Activated platelets have also been shown to form aggregates with monocytes which have been linked to myocardial infarction [145] but also as having a protective role in ulcerative colitis, suggesting complexity in their functional roles [146].

Platelet function extends through the release of platelet EV (PEV). PEV are often cited as the most abundant circulating EV with a subpopulation of PEV responsible for the properties of “platelet dust” [6]. High surface area is critical for PS activity, so in addition to platelet PS externalisation, PS-expressing platelet-derived EV (PEV) are generated and released [147, 148]. The physiological importance of externalised PS on PEV is demonstrated by Scott syndrome, a rare and mild bleeding disorder caused by a homozygous mutation in the TMEM16F/ANO6 gene on chromosome 12q12 [149]. Platelets and PEV from a mouse model of this disease have reduced levels of externalised PS and therefore fail to potentiate activation of clotting factors (e.g. FX) [150]. Despite the clear defect in PS externalisation from Scott syndrome platelets, they successfully undergo apoptosis and form PS⁺ apoptotic bodies [151], further highlighting cell-cell variation in EV biogenesis [152, 153].

PEV have been found to mediate platelet roles, often showing superior function than platelets alone, for example in a rodent model of uncontrolled bleeding, PEV administration was more successful than platelets for maintenance of arterial blood

pressure, prevention of metabolic acidosis and reduction in blood loss [154]. PEV may also exhibit procoagulant properties independent of provision of a negatively charged surface, through direct binding of fibrinogen in a $\alpha_{IIb}\beta_3$ -dependent manner [155]. PEV have also been shown to be partially responsible for the procoagulant properties of fresh frozen plasma (FFP), with differences in PEV content potentially affecting clinical outcome [156]. The EV profile of FFP is particularly sensitive to preparation variables such as anticoagulant choice, processing time and temperature [157-159]. Physiologically, PEV are also released upon traumatic injury where both low and high levels have been independently associated with survival [156, 160]. PEV have further roles in clotting and coagulation through $\alpha_{IIb}\beta_3$ mediated fibrinogen/fibrin binding where they are incorporated into clots and aid in platelet clot integration [161].

In addition, PEV have also been shown to exhibit several non-haemostatic effects including, inflammation and modulation of vascular tone which may be beneficial for the treatment of haemorrhage/trauma [154, 162]. PEV roles in inflammatory responses are mixed and potentially context dependent. PEV presence increases IL-6 and TNF- α generation from circulating cells in response to LPS treatment as shown by Balvers *et al* where depletion of EV resulted in a reduction in inflammatory response to a relevant stimulus [163]. PEV monocyte aggregates also form which can transfer platelet protein such as GpIb to monocytes and promote monocyte recruitment to the blood vessel wall [164]. Nitric oxide (NO) is implicated in endothelial-mediated blood vessel regulation and inhibits platelet aggregation [165]. PEV have also been shown to prevent phosphorylation of endothelial nitric oxide synthase (eNOS) and thereby reduce NO synthesis which reduces NO-mediated platelet aggregation inhibition

[166]. PEV may also directly be involved in reestablishing vascular integrity following vascular injury, with the likely mechanism through induction of phenotypic changes in endothelial cells [167]. PEV roles in the vasculature are shown in Figure 1.6.

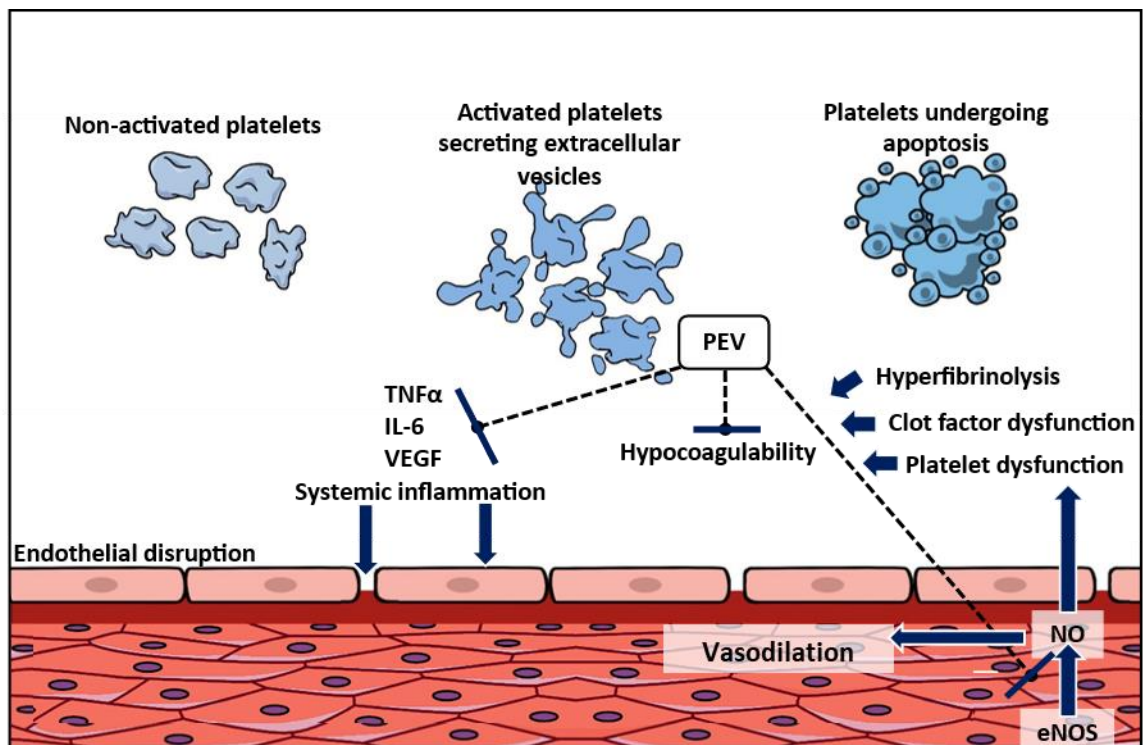


Figure 1.6. Potential platelet derived extracellular vesicle (PEV) roles in the vasculature.

PEV have roles in modulating inflammation, endothelial disruption, combatting hypocoagulability, reducing vasodilation through inhibition of endothelial nitric oxide synthase. Original figure made using mindthegraph.com.

However, despite the increasing evidence base, the procoagulant properties of PEV have yet to be fully explored clinically even though they may hold advantages over platelet transfusion alone. Pre-clinically, results are mixed. Initially, canine models of thrombocytopenia treated with remnants of sonicated platelets, or as Hjort termed them “disintegrated platelets”, had minimal effect on clotting time, large increases in prothrombin consumption and caused considerable distress [168]. However, later work suggested clinical efficacy of, what were then termed infusible platelet membranes (IPM) on thrombocytopenic rabbits [169]. In vitro models of thrombocytopenic human blood under flow conditions have also been treated with IPM and were shown to be haemostatically effective [170]. Human trials of IPM were conducted in the late 1990s, phase I trials suggested safety, with minimal risk of immunogenicity [171]. Phase II trials also suggested the preparation was safe, but failed to establish efficacy in the cohort of thrombocytopenic patients [172]. The phase II trials selected bleeding time as the primary outcome, which can remain elevated even when haemostasis is achieved [173]. PEV preparations used in human and animal studies involve diverse methods of generation, often incorporating isolation and resuspension of MV in a balanced salt buffer prior to infusion and are primarily aimed at treating thrombocytopenia. Furthermore, these preparations often include large platelet remnants in addition to PEV. One publication suggests a PEV rich platelet lysate generated via sonication and suspension in a balanced salt solution could be used as a topical treatment for bleeding in trauma patients [36]. Interestingly, Human platelet lysates (HPL) are generated via freeze-thaw cycling of expired platelet units and contains high levels of growth factors and form an alternative to foetal bovine serum (FBS) for the expansion of a diverse range of cell types [174, 175]. HPL contain

fibrinogen, so in the presence of calcium form aggregates. This aggregate observation is demonstrated in early-generation HPLs requiring the use of heparin within cell culture media. Next-generation HPLs are marketed as not requiring anticoagulant addition and are likely to utilise a process to remove fibrinogen. HPL EV content has not been assessed but it is feasible that a similar HPL process could produce a PEV rich and acellular alternate resuscitation fluid to platelets and maybe even plasma. Platelets for transfusion are stored at room temperature under gentle agitation and have a shelf life of 5-7 days. Stringent storage conditions leads to high wastage and demand for constant acquisition [176]. Platelets have been described as essentially unavailable in remote and austere environments [177-180]. The PROMMT study, a multicentre prospective observational trial of civilian trauma, highlighted that increased use of plasma or platelets relative to packed red blood cells (PRBC) was associated with a reduction in 24-hour mortality [181]. PEV in plasma may combine the benefits of platelets and plasma without the stringent short shelf-life potentially capable of being freeze-dried or lyophilised as is already happening for some plasma resuscitation fluids [182-185].

1.3.5 Platelet or Megakaryocyte EV?

Although it is claimed that PEV are the most abundant circulating EV population in the blood many researchers have failed to note or ignore the potential of megakaryocyte (MK) derived EV as a major contributor to this pool. It has been demonstrated that MK EV can be generated and released into the circulation during thrombopoiesis [186]. Although this is perhaps unsurprising, the large circulating EV population is often associated with platelet dust and is characterised based on its functional contribution

to thrombin generation. In practice, it can therefore be difficult to differentiate or distinguish platelet EV from MK EV [187]. As platelets and MK express a large panel of identical glycoproteins there are few if any specific EV markers available. Furthermore, candidate markers can be difficult to validate as MK are rare cells and difficult to handle. MK *in vitro* cell culture requires induced pluripotent stem cell (iPSC) reprogramming or MK isolation and differentiation [188, 189].

However, despite this, some candidate markers have recently been identified. In 2009, the Italiano group published a paper hypothesising that PEV are only generated following platelet activation and are therefore most relevant in cardiovascular disease (CVD) as biomarkers [186]. However, platelet activation here is not clearly defined and may involve a range of different mechanisms, sustained calcium signalling is likely necessary for PEV generation, but may not need to be as sustained for other forms of platelet activation [2].

They suggest that in the circulation in health, that the PEV population is therefore likely to be predominantly MK derived and not from platelets. Italiano suggests that when significant platelet activation occurs PEV begin to appear systemically in the blood. This is important as if specific PEV markers can be identified they will therefore provide more highly specific biomarkers of platelet activation in disease and remove the background signal/noise provided by from MK EV. Italiano identified full length filamin in MK EV and absence of CD62P and lysosome-associated membrane protein-1 (LAMP-1). In contrast, PEV from activated platelets were shown to contain cleaved filamin and were positive for CD62P, LAMP-1 and PS [186]. Italiano's initial finding of

CD62P negative release has been revised and is now described as low CD62P expression with respect to PEV CD62P expression [190].

Separately it was also shown that platelets activated with collagen-related peptide lose GPIIb/IIIa expression through enzymatic cleavage but retain CLEC2 [191]. This could therefore provide an additional specific marker of PEV as MK EV would, by definition, be GPIIb/IIIa positive. A recent review showcased a panel of markers that are likely specifically localised to platelet and MK EV or common to both (Figure 1.7) [187].

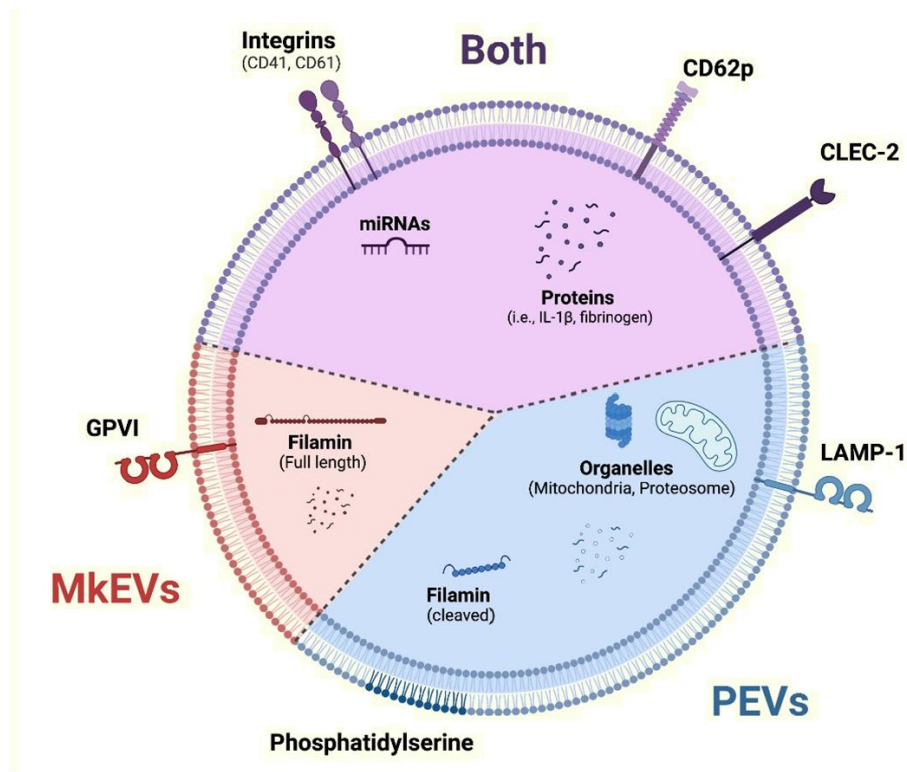


Figure 1.7. Megakaryocyte (MK) extracellular vesicle (EV) and platelet derived extracellular vesicle (PEV) biomarkers.

Specific MK and PEV expressed markers are shown in red and blue respectively. Shared MK EV/PEV markers are shown in purple. Figure taken from [187].

1.3.6 Tissue Factor and EV

Despite increased understanding of haemostasis, there remains controversy and inconsistencies surrounding TF [192]. Circulating TF concentration is reportedly as high as 10 nM [193, 194], but only 20 fM of circulating TF is active [195]. Even fM concentrations of TF increase clotting time *in vitro* [195]. Circulating TF is found on circulating monocytes [196], EV [197] and as a free soluble form [198]. The soluble form of TF is an alternately spliced variant of TF [198] although its measurement is confounded by similarity to TF degradation products [193]. In the absence of a membrane to aid complex formation, “free” TF maintains PAR2 cleavage capabilities but without other haemostatic functions [199]. Some inactive TF is also commonly termed encrypted [193] and through de-encryption becomes active. The mechanism underpinning TF encryption status is unknown but de-encrypted TF has been hypothesised to require the Cys¹⁸⁶-Cys²⁰⁹ bond (disulphide bridge) to be oxidised and intact [200]. However, mixed reports support and refute the relevance of disulphide bridge status relevance to TF activity as highlighted in the review by Butenas *et al* [136]. For example, there is evidence that the lipid microenvironment is also important for TF de-encryption [201]. From the beginning TF was controversial, even its original name: platelet tissue factor had to be changed. Historically platelets have been found to express TF, however currently it is largely agreed that platelets do not express TF, but this view is not supported by all groups. Initial results have been disregarded due to lack of adequate controls, cell contamination and lack of high-specificity antibodies [196, 202]. Despite this some evidence does suggest *de novo* synthesis of TF with

mRNA transcripts identified in platelets, however there is approximately an equal number of papers reporting the opposite [193].

Under pathological conditions, the concentration of TF can increase in the circulation. For TF to be active it is thought to require de-encryption through a mechanism hypothesised to require the Cys¹⁸⁶-Cys²⁰⁹ bond to be oxidised and intact [200]. TF can be found on activated circulating monocytes (*in vitro* this is achieved by LPS stimulation) [203], on extracellular vesicles (EV) [204] and as a soluble, alternatively-spliced form that has low procoagulant activity [198, 205]. There is mixed evidence to support and refute TF expression on eosinophils, neutrophils and platelets [136, 192]. One plausible explanation for the mixed evidence of TF expression on these cells is that it is purely pathogenic and is driven by monocyte TF-expressing EV release which are received by other cells, which can then exhibit an acquired TF expression [206].

TF EV can also be released into the circulation physiologically, for example by some cancers (pancreatic [207], ovarian [73, 208]) and after trauma, especially traumatic brain injury [209], where they can significantly activate coagulation. TF+ EV have also been hypothesised to be linked with thrombosis in COVID-19 [204, 210-213]. The interest in TF as a biomarker for cancers such as ovarian and pancreatic remains high, however clinical application has been slow due to the difficulty in measuring TF accurately.

Accurate and specific measurement of TF EV is challenging [214, 215]. Nieuwland *et al* [214] and Gardiner *et al* [215] highlight the specific challenges of TF EV research and suggested a roadmap toward standardising TF EV measurements. Measuring TF-expressing EV necessitates a highly sensitive measurement methodology, probably

in combination with a purification process. In the absence of a gold standard, comparison to activity assays is required [214, 215]. Pre-analytical variables are paramount and it is important to use well-accepted positive and negative controls to establish the accuracy of an assay [214, 215]. An assay that can differentiate positive and negative controls whilst correlating with other measurement methods (e.g. activity) is a candidate assay for application to clinical samples [214, 215]. Increasingly, antibody-based capture systems have been shown to have great utility in capturing EV based on their marker expression [216]. Capture based systems therefore can act as a dual purification and measurement platform, thus allowing for direct affinity capture of EV with minimal sample manipulation within complex biofluids such as plasma. Capture based systems also potentially facilitate detection of acute but biologically relevant and even rare EV sub-populations and be of particular relevance for TF positive EV. Direct antigenic measurement of TF in plasma has been reported, however, the low abundance of TF (<20fM [193], <2 pM [217]) in healthy individuals typically falls below or is near the detection limit of these assays [207, 217-219]. Furthermore, background signal is high when measured in plasma [73, 217, 220]. These issues are further compounded by the specificity of the antibodies used and the type of TF standard employed [221]. On the contrary, TF activity assays are highly sensitive [222] but typically require the isolation of EV from plasma [223]. Furthermore, TF activity assays cannot measure encrypted TF which may form a pool of physiologically latent TF activity. Despite these problems, the direct measurement of

TF in plasma may hold clinical utility to identify individuals at risk of thrombosis and cancer [71, 197, 224]

1.3.7 Plasma

Plasma is a complex, viscous biofluid that can be derived from whole blood following a variety of protocols using different centrifugation steps and different anticoagulants, which may impact plasma components [223]. Plasma represents a heterogeneous mixture of protein, lipids, lipoprotein and a diverse range of EV with differing sizes and from various cellular origins [2, 223]. It is widely agreed that plasma is the preferred medium for measuring and isolating blood EV. Despite this, some researchers also measure EV within either whole blood or serum. Whole blood has the advantage of less pre-analytical variables but is disadvantaged by the continued presence of cells which may alter any measured EV profile, both through *ex vivo* EV release and uptake. Serum is widely used for measuring many biomarkers including EV, but clotting may induce significant changes in EV release from blood cells and/or incorporation and loss of EV into the clot which is eventually removed by centrifugation [225]. Ultimately the choice between plasma, serum or whole blood is influenced by the research question being asked and the EV population of interest being measured.

1.3.6.1 Blood collection

Different gauge needles can affect the shear force experienced by blood, with high shear force generated by smaller gauge needles potentially promoting platelet activation and PEV generation [226, 227]. Tourniquet use can also promote platelet activation through blood stasis and should be loosened or removed after blood has

started being collected [225]. The venipuncture should ideally be uncomplicated with a clean draw and the first draw of blood is more likely to contain vessel wall cell contamination and activated platelets and immune cells so it is recommended to discard the first tube collected [228]. Following blood collection, it is important that the tubes are optimally filled with the correct volume of blood and the anticoagulant is immediately well mixed with the sample. To avoid platelet activation and EV release blood tubes should be inverted gently 8-10 times and not shaken [229]. Transport, temperature variation and delays prior to centrifugation may also promote EV release so must be standardised and ideally kept short [229, 230]. Time of day of blood collection is also an important factor shown to affect EV concentration more than exercise and stress [231]. Fasting status is also likely to affect EV, although the direct effect on EV is unknown, it has been repeatedly shown that chylomicron and lipoprotein concentrations increase, and this itself can cause issues with EV isolation [232, 233].

1.3.6.2 Anticoagulants

Different anticoagulants can impact EV and their analysis in plasma. Although heparin will maintain physiological divalent cation levels it is usually not recommended for EV research as it can have potential negative impact on EV due to its charge and potential binding to cells, platelets and EV. Heparin has also been shown to reduce the platelet activation threshold and promote artefactual PEV release. Strong calcium chelators such as ethylenediaminetetraacetic acid (EDTA) can also promote changes in cell size and activation and cause EV-platelet binding and changes in EV count [234]. Weaker calcium chelators such as tri-sodium citrate reduce EV the count to a lesser extent,

but this is often in comparison to heparin anticoagulation which can be artificially elevated [235]. The International Society for Thrombosis and Haemostasis (ISTH) vascular biology scientific and standardisation committee (SSC) recommends using trisodium citrate anticoagulation for EV work [236].

1.3.6.3 Platelet contamination

Plasma contains significant residual platelet contamination, which has long been recognised as leading to an artefactual increase in PEV concentration [229, 236]. Depending on the EV population being measured this may not matter but in the context of this thesis with measurement of platelet and procoagulant EV this is important. The ISTH SSC recommend the use of double centrifugation (2500 g for 20 minutes, twice, alternatively 2000 g for 20 minutes once, followed by 13,000 g for 2 minutes) to generate PFP as opposed to PPP [236]. Berckmans *et al* shows that the presence of artefactual PEV leads to increased thrombin generation and is proportional to the concentration of platelets prior to freezing but this can also vary with anticoagulant use and other preanalytical variables [144]. However, it has also been recently questioned to how platelet-free PFP really is. Unsurprisingly, some authors have shown that residual platelets still remain following double centrifugation at a concentration undetectable by conventional whole blood analysers but detectable by flow cytometry [237]. Recently, it was shown that plasma filtration through a 0.8 µm filter further reduces platelet contamination and the authors depicted a model that suggests this will reduce PEV contamination in freeze-thawed samples [237]. Rienk Nieuwland's group therefore recommend double centrifugation of plasma followed by filtration,

especially for miRNA EV where they show considerable reduction in total miRNA with reduced platelet contamination [237, 238].

1.3.6.4 Plasma EV contaminants

Part of the EV plasma measurement challenge is due to the requirement of EV isolation for most downstream analysis and the rich source of contaminants that can co-isolate with EV. For example, plasma protein concentration is approximately 70 mg/ml with 60% of that albumin and the remaining 40% made up of immunoglobulins, clotting factors, fibrinogen and other proteins [239]. Protein can bind to EV via electrostatic interactions, lipid-protein, or specific protein-protein interactions, form aggregates, co-precipitate or co-filtrate with EV dependent on method of isolation used [223]. Protein can also be introduced to the sample from cells and cellular debris, which can be induced by improper plasma preparation and storage conditions [2]. This is part of the reasoning behind avoiding the use of serum, which involves activating platelets and clotting whole blood, however in this case the additional EV release from activated platelets leads to increased EV concentrations [240]. Protein contamination can be somewhat avoided through the use of density-based methods of separation, but plasma also contains lipoproteins at a much higher concentration than EV (estimated at 10^{16} particles per ml [241] compared against an estimated EV concentration of 10^{10} EVs per mL [242]). Lipoprotein can also co-sediment with EVs due to their similar buoyant densities [243].

1.4 EV isolation methods

EV are found ubiquitously across all biofluids, including cell culture supernatant, urine, plasma, serum and cerebrospinal fluid. Different biofluids represent differing complexities and problems so EV isolation is often required prior to accurate measurement. However, different isolation methods offer distinct advantages and disadvantages but there is no perfect isolation method. Here I discuss the range of isolation methods currently used in EV research with Figure 1.8 showing a summary schematic of the different isolation methods.

1.4.1 Differential ultracentrifugation

Differential ultracentrifugation (dUC) is the most commonly used EV isolation method [244]. Through several rounds of centrifugations, EV are gradually separated from the remaining sample. There is no “best” protocol, but most dUC protocols start with a low g (400-2000 g) centrifugation step to remove any cellular or debris contamination. Some protocols will then centrifuge supernatant at approximately 10,000 g to remove any remaining aggregates and larger EV. This is then followed by centrifugation at approximately 100,000 g to pellet EVs where they can then be resuspended in an appropriate buffer. dUC is advantageous for concentrating EV samples, but disadvantages include the formation of EV aggregates with significant protein contamination [245]. EV purity can be improved by introducing additional EV centrifugation steps, however this comes at a price of decreased yield [246]. Sample viscosity can also be problematic- serum, plasma, synovial fluid and other viscous

biofluids require different dUC protocols to maximise yield, sometimes including longer centrifugation times with dilutions of the biofluid often required to reduce viscosity [247-249]. dUC is also time consuming, operator dependent and sensitive to instrument specification, including rotor size, and rotation radius [250].

1.4.2 Density gradient ultracentrifugation

Density gradient ultracentrifugation is an advancement on dUC that separates EV based on density (1.15–1.19 g/ml density) [246]. There are 2 major versions of this: 1) floatation gradients and 2) one-phase cushion gradients [246]. Floatation gradients are more complex and involve placing the EV containing samples at the top or bottom of graduated gradient medium composed normally of iodixanol or sucrose. Components of the sample mixture, including EV, rise or sediment to the point of the gradient equal to their buoyant density [246]. One-phase cushion gradients require placement of the EV containing samples on top of a 30% sucrose in D₂O solution. Upon centrifugation dense contaminants such as protein aggregates sediment while EV remain on top of the sucrose gradient. Due to improved separation of contaminants, density gradient centrifugation yields higher purity EV than dUC [243]. Differential gradient separation can also differentiate distinct EV populations based off their density [251]. EV obtained through density gradient methods should be washed to remove any of the dense solutions used. A disadvantage of gradient density methods is particles of similar densities such as lipoproteins can contaminate the EV layer, this is particularly problematic with plasma samples [252]. As with dUC, this method is operator dependent, and time-consuming.

1.4.3 Size-exclusion chromatography

Size-exclusion chromatography (SEC) isolates EV based on their size. EV containing samples are run through a porous gel polymer (most commonly sepharose CL-2B [253]) which is the stationary phase of the column [254]. The sample is applied by diluting with the mobile phase, which is normally sterile filtered phosphate buffered saline (PBS). The size of EV allows them to pass through the column quickly, exiting near or after the void volume; whereas smaller molecules such as free protein and some lipoprotein are slowed by the pores of the stationary phase and are eluted from the column later [255]. However, when working with plasma, chylomicrons, low density lipoprotein (LDL), and very low density lipoprotein (VLDL) can often be isolated alongside EV [256]. SEC is also available commercially through the company Izon (Christchurch, New Zealand). Izon's automated fraction collector (AFC) has addressed concerns of operator dependence and report improved reliability through standardisation of column pore size and stationary phase composition. Before this standardisation, SEC was often performed with self-made columns that were not reproducible. A disadvantage of SEC is the dilution of the resultant EV [257]. However, the resultant EV fractions are suitable for concentrating by other methods, such as dUC. Dependent on sample type, SEC isolated EV are pure, intact and can be used for functional/screening research [257].

1.4.4 Ultrafiltration

Ultrafiltration is another size-based EV isolation method. EV containing samples pass through membranes with pores of differing sizes; like SEC, this allows EV through and slows the passage of proteins and other small molecules [258]. Ultrafiltration is relatively simple but is limited by filter plugging, especially by viscous, protein dense samples such as plasma [254]. Co-retention of unwanted proteins of similar size can also be problematic [259]. For instance, this protein contamination considerably complicates subsequent analysis of the resulting EV samples by proteomics [260].

1.4.5 Polymer precipitation

Polymer precipitation of EV relies on hydrophilic polymers, most commonly polyethylene glycol (PEG) to decrease solubility of EV [261]. Although compounds such as sodium acetate and protamine have also been used for EV precipitation [262]. This causes EV precipitation where they can then be centrifuged at low speed (~1000 g) with a bench top centrifuge. The pelleted, precipitated EV can then be resuspended in sterile filtered PBS. For EV extraction, polyethylene glycol (PEG) has historically been the most used precipitating agent. PEG kits are commercially available making them particularly convenient for non-specialist EV laboratories. However, they co-precipitate protein, lipids and extracellular nucleic acids, reducing their compatibility for proteomics and RNA sequencing [256]. PEG is also difficult to remove from the resultant sample, which limits suitable detection methodologies and can negatively

impact EV function [263]. PEG precipitated samples are also difficult to resuspend, leading to significant variation in yields.

1.4.6 Affinity isolation

Affinity isolation comes in many forms and the basic premise is to capture EV using specific parameters including, membrane composition and surface markers. The most common affinity isolation method is based on immunoaffinity. There are multiple versions of this, but they typically involve beads coated with antibodies against tetraspanin markers [264], although peptide coating has also been used [265]. In many cases the beads used are magnetic and following sample incubation, the beads can be recovered and the EV removed [266]. Some authors show the use of magnetic beads coated with multiple tetraspanins to increase the depth of EV recovery obtained [267]. TIM4-coated magnetic beads have also been used to specifically isolate PS-expressing EV with the additional benefit that switching from a calcium containing to non-calcium containing buffer releases the captured EV [268]. Lectins have also been used for a more general EV capture with solanum tuberosum lectin particularly effective. Membrane sensor curvature peptides have also been used for this purpose [265]. Immunoaffinity isolation can be relatively quick and result in pure EV populations. This can be useful when looking at EV with a specific marker at low abundance. However, immunoaffinity is an expensive approach that can be limited by the quality of the antibody used and the ability to successfully detach EV [269]. Many elution buffers are acidic and can denature EV surface markers [270].

1.4.7 Microfluidics technology

Microfluidics is one of the most promising emerging EV isolation methods. They require very little sample and purification occurs within micro-sized channels that use a wide range of principles to isolate EV including immunoaffinity capture, filtration and next-generation filtration including acoustics. In theory microfluidic devices can be made that are compatible with downstream measurement process, however these devices are still in their infancy [271].

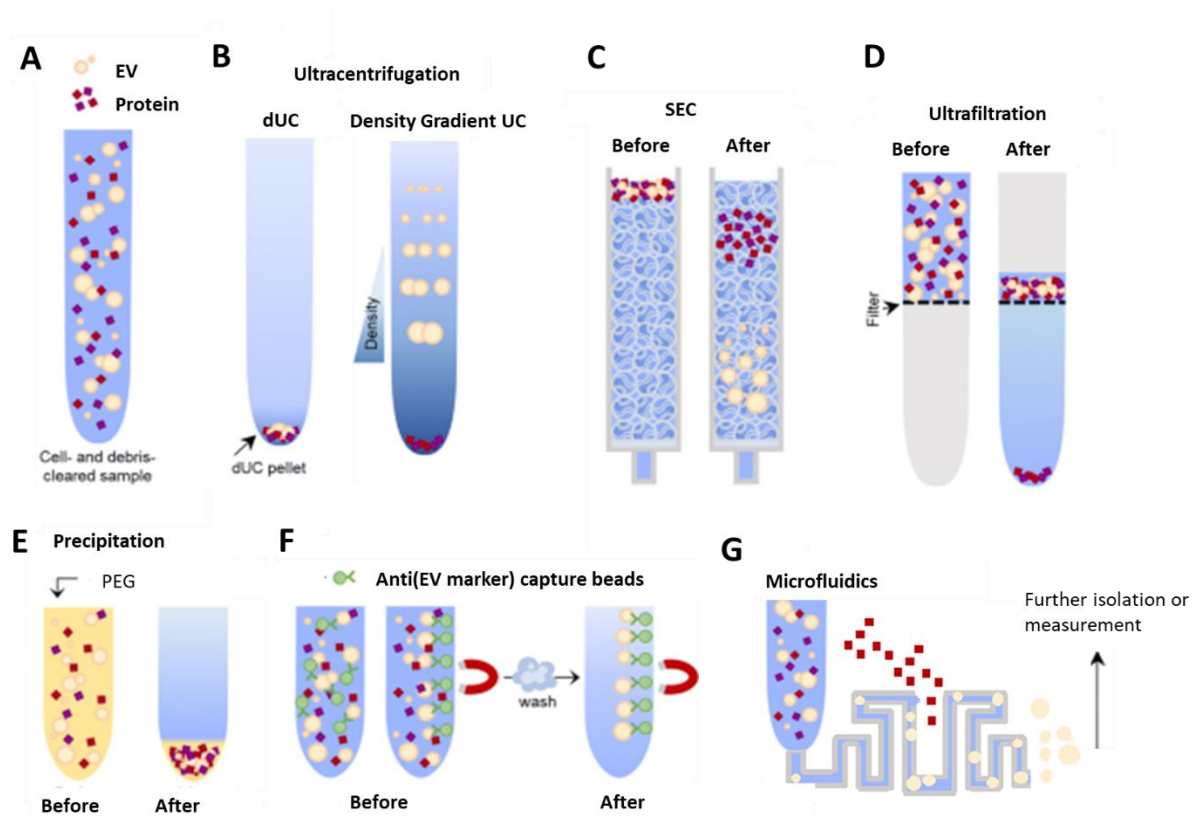


Figure 1.8. Methods of EV isolation.

Schematic representation of EV isolation techniques with red squares representing protein and yellow circles representing EV. a) A sample ready for isolation, this has occurred through cell and debris removal by low-speed centrifugation and represents the starting point for the following different isolation methods; b) Differential ultracentrifugation; c) Size exclusion chromatography; d) Ultrafiltration; e) Precipitation; f) Immunoaffinity isolation; g) Microfluidic isolation. Figure adapted from [257].

1.5 EV measurement techniques

The study of EV has been historically challenging. Different biological fluids undergo different pre-analytical purification steps, coupling this with the lack of standardisation in isolation protocols leads to highly variable samples for downstream analysis. Furthermore, there is still no gold standard EV characterisation method. The lack of a gold standard is, in part, due to the small size of EV which limits the use of conventional detection methods. In this section I will now discuss the commonly used EV measurement methods historically used in this field.

1.5.1 Electron microscopy

EVs were first visualised by electron microscopy (EM) [4, 6, 272]. Conventional light microscopy uses visible light and a series of lenses to visualise samples, whereas EM uses a focused beam of accelerated electrons that allows visualisation of nanoscale structures such as EV [272]. There are multiple types of EM, including transmission EM, scanning EM and cryoEM [272]. EM is useful for confirming that EV are present, based on characteristic morphology of EV, which differs dependent on the type of EM used [272]. Many forms of EM also have immunoaffinity compatibility allowing for the presence of EV with specific markers to be positively confirmed [273]. However, EM does require laborious and time-consuming preparation, largely cannot be used to image EVs in their native state, has limited quantification ability and is highly operator dependent [274]. Yuana *et al* show that cryo-EM can be performed on plasma samples without the need for EV purification, but images generated in this way also show lipoprotein [275].

1.5.2 Transmission electron microscopy

Transmission electron microscopy (TEM) is the most common type of electron microscopy for EV imaging [276, 277]. EV containing samples are fixed and dehydrated. Samples are then embedded and sliced in to thin (nm) sections and mounted on grids for imaging. The electron beam used in TEM can be diffracted or transmitted by the mounted sample. Bright-field images can be made from the transmitted electrons, whilst diffracted electrons can generate dark-field images that provide greater contrast. TEM is compatible with immuno-gold labelling, where EV with specific markers can be captured and visualised. EV often appear with classical cup-shaped morphology in TEM imaging, this is due to sample dehydration [175]. This has been used in confirming presence of cancer-associated markers e.g. carbonic anhydrase IX on urinary EV [278].

1.5.3 Scanning electron microscopy

Scanning electron microscopy (SEM) again involves fixing and dehydrating EV containing samples. Samples are then coated with a thin (2-10nm) conductive layer (gold/carbon). The EM electron beam can then scan the surface of the sample and generate topographical information. With SEM, EV drying causes EV collapse and characteristic saucer/round morphology to be observed [279, 280]. SEM does not require the embedding and sectioning process of TEM which reduces some sample preparation complexity [272]. However, SEM quantification is also limited and EV sizing can be overestimated due to the presence of the conductive layer and also the artefactual “coffee ring effect” [281].

1.5.4 Cryo-electron microscopy

Cryo-electron microscopy (cryo-EM) requires fixing of samples through liquid ethane cooling, this prevents ice crystal formation and vitrifies water instead. This type of fixing retains EV in their native hydrated state and thus removes the artefact of cup-shaped, or saucer shaped morphology often seen with TEM and SEM [280-282]. Cryo-EM is also compatible with immunogold labelling techniques and if desired can be followed up by fixing, embedding and dehydrating of samples for alternate EM methods to be deployed [283]. Cryo-EM provides the highest quality imaging of EV compared to other EM methods [282]. Brisson *et al* have shown a semi-quantitative cryo-EM immunogold labelling technique that is compatible with plasma, where they show that PEV released from activated platelets express CD63 ubiquitously whilst CD41 and PS are expressed in heterogeneous populations of PEV [283].

1.5.5 Atomic force microscopy

Atomic force microscopy (AFM) generates topographic images of samples and unlike most forms of EM can measure samples in a liquid state [284]. AFM can also measure dried EV [284]. Samples for AFM must be secured on an atomically flat surface. AFM involves the use of a silicon or silicon nitride probe that can probe the surface of a sample, the tip of the probe can be used in two ways: contact or tapping mode [285]. In contact mode a probe scans the surface of the sample, causing damage to sample and probe. In tapping mode, the probe oscillates and taps across the surface of the samples, thereby reducing sample-probe contact and reducing damage. In either AFM mode, the interaction between probe and sample surface leads to deflection of a

cantilever which is recorded by a laser and a photodiode [284]. This data can be used to construct 3-D images of surface topography and size EV [284]. AFM can also utilise silicon probes with antibody attached at the end, this allows for detection of specific EV markers of interest [286]. Another version of AFM involves using a flat surface to antigenically capture EV with specific markers and then perform AFM [287].

1.5.6 Dynamic light scattering

Dynamic light scattering (DLS) determines a particle's size from fluctuations of the scattered light resulted from the Brownian motion of the particles [284]. The intensity of the scattered light is proportional to the square of the volume of the particle. Small amounts of large aggregates or dust particles can disturb the size determination if the main population is significantly smaller in size. DLS is useful for measurement of monodisperse particles capable of resolving particles as small as 1 nm. However, most EV preparations have polydisperse distributions, this leads to poor resolution and inaccurate results when using DLS [288].

1.5.7 Nanoparticle tracking analysis

Nanoparticle Tracking Analysis (NTA) measures the Brownian motion of particles and can therefore be used to measure size and concentration using the Stokes-Einstein equation. NTA can measure particles from 30 - 1000 nm, with the lower detection limit being dependent on the refractive index of the particle [289]. However, NTA cannot discriminate between EV and other small particles within the biofluid such as chylomicrons and lipids [290]. NTA has been reported as highly operator dependent

and instrument dependent. This is unsurprising as there is a host of available settings to modify, for video capture alone settings include: camera gain (0-680), camera shutter speed (0.47-50ms) and camera level (1-16) [290]. Following video capture, analysis settings are also numerous including: detection threshold, which determines the minimal intensity required to qualify as a particle to be tracked; blur, which helps smooth noise, such as diffraction rings and minimum track length, which dictates how many frames a particle must be continuously tracked in order to form part of the sizing dataset [290]. Many of these settings can be automated, but detection level and camera level have a large effect on the measured outcome and are determined by the user [290]. These factors require optimisation and consistency when comparing samples within an experiment setup. In order to ensure robust results, at least 5 one minute video recordings are recommended to be made and analysed [290]. Many authors do not report the settings used for data acquisition or analysis or indeed whether they have followed the replicates suggestion [2]. NTA measurements can also be disrupted by vibration, which introduces noise. Air bubbles are a recurring issue within the flow cell of NTA devices, these lead to sample drift, intense background scattering and inaccurate results [290]. NTA flow cells are also susceptible to adhesion of protein and other material that lead to high background, flushing the system with filtered PBS or distilled filtered water can sometimes dislodge material; but in many cases the flow cell chamber must be dismantled and carefully washed by hand [290].

NTA does have some capacity to measure specific sub-populations of EV based on marker expression via fluorescence mode. However, the efficacy of this is often reported as being low [290]. The fluorescent signal needs to be very bright in order to

be detected by any NTA system [291-293]. The use of dyes that stain the lipid bilayer, such as PKH27 and PKH67 have been shown to have some success with NTA. However, use of fluorophore-conjugated antibodies has been largely unsuccessful, except for highly expressed EV markers such as CD63 [291]. However, even in the case of highly expressed EV markers, fluorescent quantification is hampered by fluorescent particles moving in and out of focus and fluorophores are susceptible to photobleaching. Q-dots are very bright fluorophores that have been shown to overcome some of these issues, but their use is disadvantaged by high background originating from unbound Q-dots and aggregates [290]. NTA does yield higher resolution than DLS and for measurement of polydisperse samples containing EV [289]. NTA also has a lower limit of detection than DLS allowing for detection of EV even at low concentrations [289]. There is a newer model of NTA called NanoSight Pro, that is reported as reducing operator dependence further by reducing the range of user-controlled setting instead relying on artificial intelligence (AI) directed camera level and focussing.

1.5.8 Flow cytometry

Flow cytometry is a powerful analytical tool that has primarily been used for single-cell analysis, cell sorting and phenotyping both cell surface markers and cargo in target cells. Flow cytometry relies upon hydrodynamic focussing of cells to form a focussed beam of cells through a flow cell illuminated by various lasers. The light scattered (forward scatter and side scatter) from particles passing through the laser at the interrogation point provides information on the physical characteristics of the particles, including size and granularity, respectively. Samples can be fluorescently labelled with

dyes or antibodies conjugated to fluorophores, the emission spectra this generates provides information on the surface marker expression and biochemical composition of particles passing through the laser beam. The vast majority of EV fall under the detection level of conventional flow cytometry (~300nm diameter) leading to measuring events that may be “noise” [294]. However, there are methods to increase the reliability of EV data derived from flow cytometry.

Next generation flow cytometers have been utilised to improve the resolution of small EV. In particular the use of fluorescent triggering or lower wavelength lasers has also improved small EV detection. Shorter wavelengths allow for better resolution of EV, side scatter is preferentially used for EV flow cytometry and combining this with lower wavelength incident lasers (i.e. 405nm (violet)) improves small particle resolution [295]. Other quality control procedure including positive (EV standards and quality control materials) and negative controls (e.g. EV lysis to show specific signals) and calibration of gating using beads of known size can also be used [296].

One such example is the CytoFLEX flow cell which was designed to maximize light capture for smaller particles. Brittain *et al* used CytoFLEX and demonstrated that by coupling specially designed flow cytometers with simplified Mie theory calculations incorporating data from polystyrene or silica beads of known size they can detect, quantify and size EV based on their refractive index (RI) [297]. However, despite this seeming advancement, the oversimplification of Mie theory and the implicit assumption that EV have a homogenous RI may overestimate light scattering signals of EVs while underestimating their diameter [298]. Van der Pol *et al* shows how modifying Mie theory calculations can improve results [298]. Nonetheless, these

methods are difficult and time-consuming and newer techniques have been developed, one promising continuation of flow cytometry are dedicated EV flow cytometers such as NanoFlow cytometers are increasingly being used to address this [299]. NanoFlow cytometers have furthered the resolution of particles all the way down to 30nm and have completely foregone the ability to measure particles above the size of 800 nm. Another promising EV measurement technology is imaging flow cytometry which works in a similar way to flow cytometry with the addition of allowing images of every event acquired, the limits of detection are similar to flow cytometry with a minimum of three pixels required to positively identify particle presence which means small particles may still not be resolved [300].

1.5.9 Tuneable resistive pulse sensing

Tuneable resistive pulse sensing (TRPS) is the use of an electric field applied to EV containing samples whilst monitoring particle movement through a nanopore. In practice, this requires the nanopore to form a non-conductive elastic membrane that separates EV from particle-free electrolyte. Upon establishing an electric current a baseline is formed, as particles (EV) pass through the nanopore they can be measured via the coulter impedance principle with the current blockade magnitude being proportional to the volume of the particle. Calibration of the current blockade to beads of known size allow for EV sizing measurements to be performed and concentration can be calculated provided volume of material measured and the rate of current blockade events. Izon have largely cornered the TRPS market with the Exoid device and utilise different diameter pores to measure from sEV to IEV. TRPS is useful but suffers from blockages of the nanopores used which can be time-consuming to

resolve. TRPS also struggles to measure particles across the range of EV sizing [301]. As EV form a polydisperse size distribution they often lead to severe blockages of the nanopore [302].

1.5.10 Omics

In addition to measuring EV, there is growing interest in measuring EV components. Omics approaches, including RNA sequencing, mass spectrometry and lipidomics can provide a wealth of data that can aid in deeper phenotyping of EV and may hold particular utility in identification of disease specific biomarkers. For example, HER2+ and triple negative breast cancer patients can be stratified based on EV proteome signatures [303]. miRNA signatures of EV can theoretically be used to identify disease-relevant miRNA that are transferred via EV and could form targets for point of care tests [304].

Currently, three public online databases of vesicular proteins, mRNAs, miRNAs, and lipids (ExoCarta [305] , Vesiclepedia [306] and EVpedia [307]) are available to be freely used.

1.5.11 Immunoaffinity

There is a clear need for a high-throughput, reliable method of single EV analysis that requires minimal pre-analytical sample purification. The newest technologies are moving towards a capture-based system, typically on silicon/gold coated chips or cartridges or alternatively incorporating immunoaffinity into existing technologies. Although, there are alternatives to chip-based EV capture methods, WhiteFox™ uses

gold probes with capture antibodies. These probes are dipped into EV containing samples and capture EV where they can then be further interrogated with secondary labelling [308].

Increasingly, antibody-based capture systems have been shown to have great utility in capturing EV based on their marker expression [30-32]. Capture based systems act as a combined purification and measurement platform, allowing for direct capture of EV with minimal sample manipulation in complex biofluids such as plasma [31]. Capture based systems potentially facilitate detection of acute but biologically relevant EV populations [33]. One promising instrument is the ExoView R100 which is the major topic of this thesis.

1.5.11.1 ExoView R100

The ExoView R100 is an instrument that utilises a customisable chip-based antibody capture system coupled with single particle interferometric reflectance imaging capability and three lasers for fluorescent detection. This allows for antigenic EV capture, followed by fluorescent labelling of up to three additional markers (LED wavelengths: 470nm, 567nm, 623nm). The principle of single particle interferometric reflectance imaging sensor (SP-IRIS) mode of the technology is the enhanced contrast in the scattering signal from nanoparticles generated using a layered substrate (silicon/silicon dioxide) [216]. ExoView R100 also makes use of a multiplex system, standard tetraspanin chips have independent antibody capture spots for CD81, CD63, CD9, each printed in triplicate. The technology underlying ExoView was first utilised in viral capture and detection by George Daaboul during his thesis work. He later turned

to EV research and commercialised the technology [216]. At the outset of my thesis this was a new instrument surrounded by a great deal of excitement. The promise of an instrument that can both isolate and measure isolated EV simultaneously from complex biofluids with no purification requirements at a single particle level was an interesting advancement. An overview of the ExoView R100 instrument is shown in Figure 1.9.

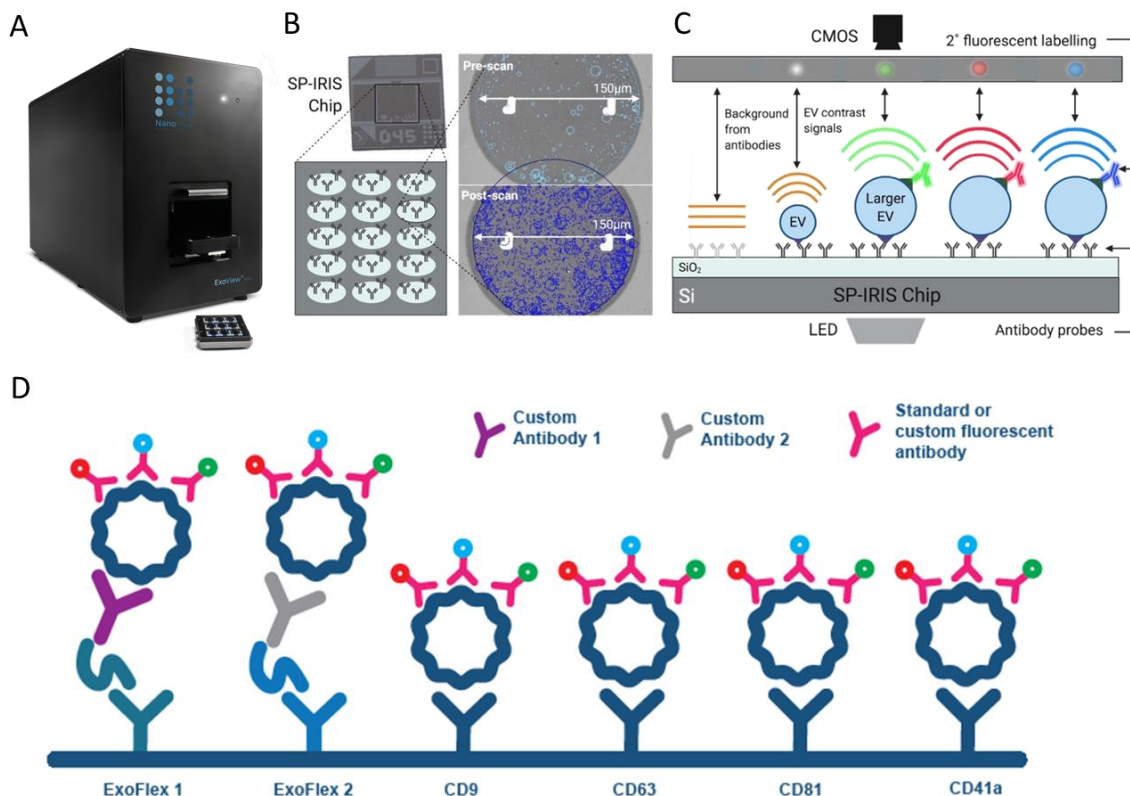


Figure 1.9. ExoView R100 method and theory.

a) The ExoView R100 instrument. b) Image of a chip, a schematic is shown focusing on the central square of the chip, where antibody or peptide material for EV capture is printed. The zoomed in schematic shows rows of antibody capture spots printed in triplicate. A further zoom shows images of the same antibody spot before and after extracellular vesicle (EV) capture. Pre scans showcase background light scatter, this occurs due to the material of the chip (silicon/silicon dioxide) and light scattered from the antibodies themselves. c) An alternate schematic of a chip highlighting the layered silicon/silicon dioxide surface of the chip and the antibodies printed on top, with EV captured. Following EV capture fluorescent labelling of captured EV can occur. The combination of an LED below the chip, a CMOS camera above and fluorescent detectors allow for both interferometric imaging data and fluorescence data to be collected. d) An alternate schematic of a chip highlighting the capacity for independently capturing EV based on both standard chips (CD9, CD63, CD81, CD41a) and ExoFlex chips which allow for customised addition of antibodies for EV capture.

1.5.11.2 ExoView Method

ExoView can be an expensive tool for EV measurement and involves some technical skill with special care required when handling chips (with tweezers) to ensure no damage occurs to the chip. However, this has been addressed with the newer companion instrument, an automated chip washer. To begin ExoView work, the first step is to load chips in the ExoView R100 reader and perform a pre-scan to account for any background, either from particles or from the inherent light scattering properties of the printed antibodies. Next, pre-scanned chips are placed in the centre of a well within a 24 well plate with the void space between wells filled with 1 ml of distilled water. Pre-diluted sample can then be added to the surface of each chip. Once chips have sample loaded on their surface it is important that they are not allowed to dry, by sealing the 24 well plate with adhesive tape plate effectively becomes a moisturised chamber that impedes evaporation of sample. It is worth noting that each chip is single use and can only measure one sample. Each chip has antibody capture spots printed in triplicate. Each spot acts an independent test interacting with only a small volume of sample. Samples are left to incubate on the surface of the chip for 16 hours overnight. The EV will settle onto the chip and bind to capture antibody if they express the antigen of interest. This effectively acts as an EV purification step potentially allowing for use of complex biofluids with no pre-isolation requirements. After incubation is complete the chips are washed to remove unbound EV and primary antibodies conjugated to fluorophore can then be added to specify co-localised markers. After sample incubation and fluorophore-conjugated antibody labelling chips are carefully moved to a 10ml petri dish filled with distilled water. The drying process

is delicate and begins with deliberate and purposeful agitation of the chips through use of tweezers. This is then followed by slow removal at a 45° angle from the petri dish. The surface tension of the distilled water should pull liquid off the chip. This is visible to the naked eye and chip drying should be restarted if this does not occur. Improper drying may lead to unreliable results. It is also worth detailing that although EV captured by ExoView can be labelled with up to three fluorescent antibodies it is difficult to compute a single EV count from the data. This is because simply totalling EV across capture spots leads to “double-counting” where EV captured on CD81 may express for example CD63 and vice versa. The ability to colocalise EV fluorescently is not perfect and it is difficult for any software to determine whether a captured EV is both red and green or whether it is two EV captured in close proximity. This is further compounded by the size limitation of light scatter (interferometric imaging) data, whereby EV smaller than 50nm cannot be counted, but in fluorescence mode these EV can be counted, but not sized. It is also feasible that different capture antibodies may have differing capturing efficiencies, to avoid any apparent EV differences due to this I recommend only comparing capture spots that use the same antibody and clone i.e. rather than comparing CD81 capture to CD63 capture within a sample the best approach is to compare CD81 capture in one group of samples compared to another.

1.5.11.3 ExoView in the literature

There are now several publications that utilise ExoView technology. One important paper primarily compares ZetaView and Nanosight NS300 (two NTA instruments), interestingly they also include comparison with ExoView and other EV technologies. Unsurprisingly, ExoView provided concentrations of EV were lower than with

NanoSight NS300 and Zetaview. The reason for this reduced concentration with ExoView, is due to three major factors: 1, NTA instruments overestimate concentration as they measure all particles present, not just EV; 2, ExoView captures EV based on marker expression, so EV will only be recorded if they are bound, and 3, there is no effective way to summarise the concentrations measured by ExoView e.g. totalling each capture spot will lead to double counting of EV that express both markers (See Figure 1.10). By double counting I mean that each antibody capture spot can be thought of as independently capturing EV and each spot only interacts with a small volume surrounding them. The surrounding volume of each spot should be identical to every other spot in terms of the proportions of EV with different markers (i.e. the sample is well mixed prior to chip application). Therefore, if there is a population of EV that are CD81+ CD63+ this population will be represented on both CD81 and CD63 capture, so totalling these spots will lead to some amount of double counting.

Interestingly the size of EV measured by ExoView was also lower than both NTA and ZetaView but very similar to TEM provided sizing. This is likely because the drying process used prior to reading chips shrinks EV. An alternative explanation is that ExoView software assumes a constant RI (~1.45), which may be leading to under sizing of EV (MISEV 2023 (in press)).

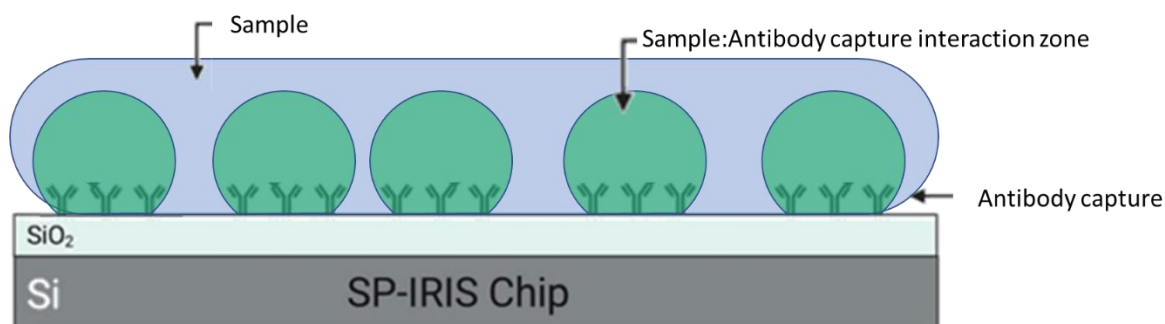


Figure 1.10. ExoView R100 schematic of sample interaction with capture antibodies.

A sample is incubated on the surface of the chip overnight for 16 hours. Each spot interacts independently from other spots. The surface area of each antibody capture spot is fixed and theoretically interacts with the same volume of sample.

Other uses of ExoView have included the formation of a miniature compendium detailing the tetraspanin expression profiles of EV from different sources [309]. Multiple papers have confirmed that platelet contamination in plasma and serum samples leads to increased PEV concentrations. Prior to ExoView, flow cytometry was the only method that could do this, but flow cytometry only captures the peak of EV size distribution. McIlvenna *et al* show that high intensity intermittent training increases the concentration of CD9, CD63 and CD81 EV in circulation [310]. They also show validation data for the plasma used in their study and show that PPP contains a higher concentration of platelets than PFP and this results in a higher measured concentration of CD41, CD9 and CD63 expressing EV. Increasingly, literature reports focus on the fluorescent element of ExoView, this now seems to be the agreed upon single best feature of ExoView.

1.6 Thesis Aims

EV measurement is notoriously difficult, yet they are crucially important in health and disease. Plasma is also a complex biofluid which exacerbates the ability to reliably detect and measure EV as potential biomarkers of disease. ExoView is a new platform that has enormous potential to address the difficulty in directly measuring plasma EV. This project was originally meant to be focussed on EV in cardiovascular disease. The original project aimed to specifically identify EV signatures by ExoView within samples from patients post myocardial infarction randomised to differing anticoagulant treatments from the observational CASCADE (Cardiovascular and bleeding outcomes associated with antithrombotic therapy) study (IRAS reference 238646). Unfortunately, I was unable to collect sufficient samples as COVID-19 led to this trial

being halted at a stage where minimal patient recruitment and follow ups had occurred. The primary hypothesis could therefore not be tested. Given these problems during the first 2 years of my project the aims were therefore changed and broadened:

The modified, specific aims of this project were to address:

- Can ExoView be used to measure single EV within plasma?
- To set up and optimise ExoView for the measurement of EV within plasma.
- Apply optimised ExoView plasma EV measurements to measure procoagulant PS-positive EV in plasma.
- To determine the potential of customizing ExoView for the measurement of the rare population of TF positive EV in plasma.
- To determine the origins of circulating EV within plasma using the ExoView. Are circulating EV derived from megakaryocytes or platelets?

Chapter 2. Materials and Methods

2. Methods

2.1.1 Volunteers

This study received ethical approval from the University of Birmingham's Science, Technology, Engineering, and Mathematics Ethical Review Committee (ERN-18-1017). Healthy volunteers were recruited from the University of Birmingham Research Laboratories, Queen Elizabeth Hospital. Exclusion criteria for healthy volunteers included a history of bleeding symptoms and use of aspirin/non-steroidal anti-inflammatory drugs (NSAIDs) and other antiplatelet drugs within 1 week. Blood was withdrawn from each participant via antecubital venepuncture using a sterile 21-gauge needle and aseptic technique. The first 4 ml of blood were withdrawn into EDTA anticoagulant BD vacutainers (Becton Dickinson (BD, Oxford, UK) for full blood counts using the Sysmex XN1000 (Sysmex UK, Milton Keynes). Up to 42 ml of blood was then drawn into tri-sodium citrate (3.2%) anticoagulant vacutainers (BD, Oxford UK) for experiments (9:1 vol/vol). A family with history of bleeding and suspected Scott syndrome were consented and recruited to the GAPP study as previously described [311] under approval of the UK National Research Ethics Service by the Research Ethics Committee of West Midlands (06/MRE07/36). Blood collection followed the same protocol as for healthy volunteers detailed above. Blood samples were collected from trauma patients during prehospital emergency evacuation, prior to receipt of prehospital blood products (research ethics committee approval: Brain Biomarkers After Trauma Cohort Study; reference 13/WA/0399). Blood samples were collected from patients with ovarian cancer using a protocol approved by the University of North

Carolina at Chapel Hill Institutional Review Board (11-1201). Some of the ovarian cancer and trauma samples were used in previous published studies [39] [40].

2.1.2 Plasma types and their generation

Platelet Rich Plasma (PRP) was prepared from whole blood, following the recommendations of the Platelet Physiology Subcommittee of SCC/ISTH by 200 g centrifugation for 10 minutes with no brake [312]. PRP was aspirated without disturbing the buffy coat. Residual blood underwent a second centrifugation at 1500 g for 20 minutes. Platelet Poor Plasma (PPP) was aspirated without disturbing the buffy coat. A vesicle rich preparation termed Platelet Enhanced Plasma (PEP) was generated by 3x freeze-thaw cycling of PRP at -80°C and 37°C. After the final thaw, the preparation underwent 3000 g centrifugation for 10 minutes to remove residual platelet remnants and aggregates and was stored at -80°C. Platelet free plasma (PFP) was generated by centrifugation of tri-sodium citrate anticoagulated whole blood at 2000 g for 20 minutes, followed by 13,000 g for 2 minutes. For some experiments, tissue factor positive control PFP was generated by first stimulating whole blood with lipopolysaccharide (LPS) (10 µg/ml) (L2887, Sigma-Aldrich, Massachusetts, US) for 5 hours at 37°C followed by double centrifugation as described above. For ovarian cancer samples PPP was generated by 1500 g centrifugation for 15 minutes. Platelet counts were performed using the Sysmex XN1000 (Sysmex UK, Milton Keynes). For some experiments platelet counts were performed by either flow cytometry and/or the Sysmex XN1000 (Sysmex UK, Milton Keynes).

2.1.3 Expired Platelet Units

A total of four expired platelet units were received from National Health Service Blood and Transport (NHSBT). Two units were 12 days old upon receipt, with the remaining two units 8 days old upon receipt. Expired platelet units were centrifuged at 200 g for 10 minutes, with no brake to remove residual white blood cell and red blood cell contamination. The top 30 ml of supernatant was aspirated, gently mixed and subjected to a complete blood count (Sysmex, UK, Milton Keynes). Separately 10 ml of platelet units were centrifuged at 2000 g for 20 mins and then 13,000 g for 2 mins to generate a platelet free plasma. Processed units were subjected to three freeze-thaw cycles to generate PEP derived from expired platelet concentrates. Full blood counts were performed upon sample receipt, following 200 g centrifugation and on PFP.

2.1.4 Platelet isolation, activation and EV supernatant collection

Platelets were isolated following the method by Alshehri *et al* [313]. Briefly 0.1 µg/ml prostacyclin was added to fresh PRP and platelets were pelleted by centrifugation at 1000 g for 10 minutes. The platelet pellet was re-suspended in acid-citrate-dextrose and modified Tyrode's buffer containing 129 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 mM glucose, 1 mM MgCl₂, pH 7.3 and 0.1 µg/ml prostacyclin was then added and the platelets re-centrifuged at 1000 g for 10 minutes. Platelet counts were performed using the Sysmex XN1000 (Sysmex UK, Milton Keynes). For some experiments platelet counts were also performed by flow cytometry as detailed under

the subheading “Platelet flow cytometry”. The platelet pellet was resuspended in modified Tyrode’s buffer to a concentration of 100×10^9 platelets/L for experiments unless otherwise indicated. Once purified, platelets were referred to as washed platelets.

Platelet activation occurred by addition of either collagen-related peptide (CRP; $10 \mu\text{M}$), ionomycin ($10 \mu\text{M}$), for 15 minutes at room temperature unless stated otherwise, then diluted 40-fold with modified Tyrode’s buffer. Platelets were analysed by flow cytometry using a FACSVia (BD Biosciences, New Jersey, US) on BD Accuri C6 Plus software (see section 2.1.15 Platelet Flow Cytometry).

2.1.4 Commercial platelet lysates

Human platelet lysates (HPLs) were also obtained from 3 different companies: MultiPL’100 (MacoPharma, Mouvaux, France), PL_{SOLUTION} Research Grade (PL Bioscience GmbH, Dennewartstr, Germany) and CRUX RUFA Research Trinova Biochem GmbH, Rathenaust, Germany). All HPL preparations were thawed at 37°C , aliquoted and stored at -80°C . HPL aliquots were thawed at 37°C for 10 minutes prior to experimental analysis.

2.1.6 Megakaryocyte supernatants

Megakaryocytes (MK) were generated and supernatants containing EV from their cell culture were kindly provided by Professor Cedric Ghevaert. MK were generated following the optimised protocol described by Moreau *et al* using forward programming (FOP) as previously described [188]. At day 10 of culture, prior to proplatelet formation, MK conditioned media was collected and centrifuged at 300 g for 5 minutes to remove

large debris and subsequently double centrifuged at 2000 g for 20 minutes and 13,000 g for 2 minutes. Cell culture supernatant sample aliquots were then stored at -80°C prior to analysis.

2.1.7 Thrombin Generation

Thrombin generation was assessed by using calibrated automated thrombography (CAT) as described by Hemker *et al* [314]. Briefly, 96 well round bottomed plates were prepared with 4 wells containing 80 µl of sample and 20 µl of either PRP reagent (Stago, UK) (5 pM tissue factor) or thrombin calibrator (Stago, UK). Other experiments were conducted where PRP reagent was replaced with MP reagent (Stago, UK) or PPP reagent (Stago, UK). In all cases, plates were then incubated for 10 minutes at 37°C, after which 20 µl of FLUCA reagent (fluorescent thrombin substrate Z-Gly-Gly-Arg-aminomethylcoumarin (ZGGR-AMC) and CaCl₂ (CAT# 86197, Stago, UK) was automatically added to all wells to initiate thrombin generation. After sample activation samples were monitored using a fluorescent plate reader (390 nm excitation, 460 nm emission, Fluoroskan Ascent, Thermo Scientific™, Massachusetts, US). Thrombin generation parameters (lag time, peak, time to peak, ETP (endogenous thrombin potential/area under the curve, start tail) were calculated by Hemker software (Thrombinoscope Software Version V5.0.0.742, Stago, Australia). In some experiments (MP reagent only), PFP samples were double centrifuged at 20,000 g to pellet and remove EV and either HTF-1 anti-Tissue Factor (TF) antibody (final concentration: 9.15 µg/mL) or control mouse IgG were added to wells prior to the 10-minute incubation period. Figure 2.1 details the CAT methodology.

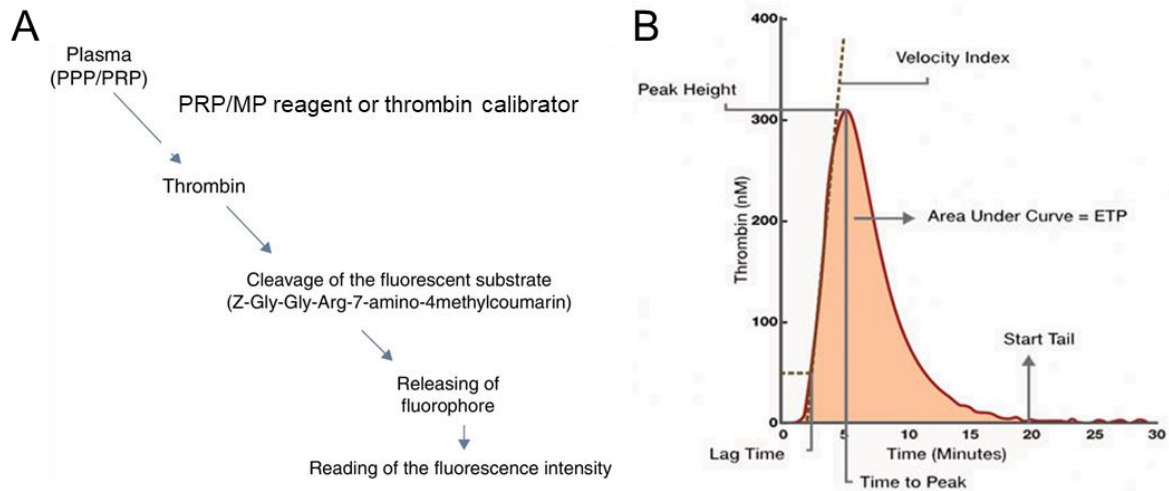


Figure 2.1. Calibrated automated thrombography (CAT) method and parameters.

a) Illustration of the method for CAT, plasma, activator reagent (platelet rich plasma reagent/ microparticle reagent) or thrombin calibrator and a thrombogenic substrate are added to wells. As thrombin is generated the substrate Z-Gly-Gly-Arg-7-amino-4methylcoumarin is cleaved, the fluorescent product of this reaction is read and can be used to monitor thrombin generation over time and analysed using Hemker software. b) A typical CAT trace with the important measured parameters shown including lag time, peak, time to peak, velocity index, area under the curve (AUC)/endogenous thrombin potential (ETP) and start tail.

2.1.8 ROTEM

Rotational thromboelastometry (ROTEM) was performed using a 4-channel instrument (ROTEM® Delta, TEM Innovations GmbH, Munich, Germany). ROTEM was used to measure clotting time (CT), clot formation time (CFT) (time to reach clot amplitude of 20 mm), maximum clot firmness (MCF) and the alpha ($^{\circ}$), which is the angle of the tangent between 0 and 20 mm clot amplitude. Each measurement channel consists of a disposable cuvette fixed in a temperature-controlled metal cup-holder and a disposable pin attached to a moving axis, stabilized by a ball-bearing. The ROTEM axis rotates back and forth at 4.75° twelve times per minute. The citrated whole blood or plasma sample in the cup is recalcified and activated by an automated pipetting programme that adds 20 µl of recalcification reagent (TEM Innovations GmbH, Munich, Germany) and 20 µl of activation reagent. The activation reagent was either INTEM (TEM Innovations GmbH, Munich, Germany) or EXTEM reagent (TEM Innovations GmbH, Munich, Germany) for investigation of the intrinsic and extrinsic pathways, respectively. Following sample activation clot strands between pin and cup-wall increasingly impede pin rotation. These changes in pin movement are detected by a LED light-mirror-light detector system, this signal is processed and transformed into a thromboelastometric curve. Instrument and sample temperature were maintained at 37°C, except for investigation of PEP treatment within an *in vitro* whole blood coagulopathy model where the temperature was reduced to 32°C to simulate hypothermia. ROTEM allows for the measurement of clotting time (CT), clot formation time (CFT), maximum clot formation (MCF) and lysis parameters. Figure 2.2 details ROTEM methodology.

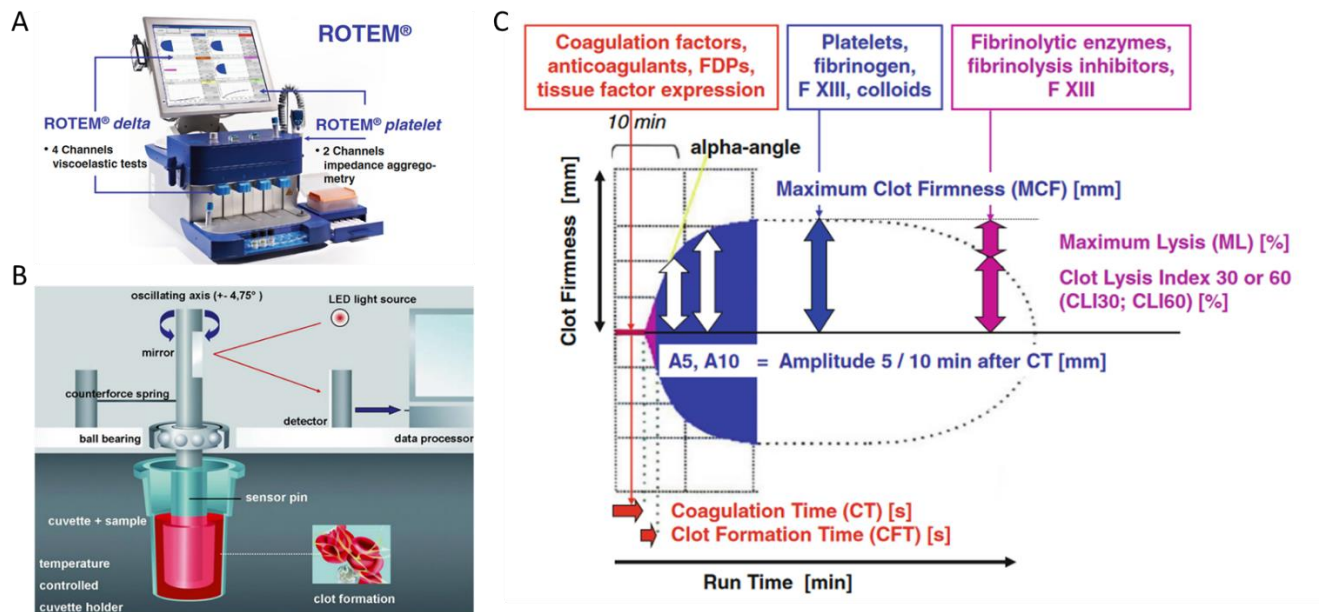


Figure 2.2. Rotational Thromboelastometry (ROTEM) method and parameters.

a) The ROTEM delta instrument. b) Principle of ROTEM measurement: the ROTEM axis/pin alternates back and forth by 4.75° twelve times per minute. Following recalcification of the citrated whole blood or plasma sample in the cup and adding an activator (EXTEM/INTEM) clot strands between pin and cup-wall increasingly impede pin rotation. These changes in pin movement are detected by a LED light-mirror-light detector system, this signal is processed and transformed into a thromboelastometric curve. c) A typical thromboelastic curve with important measured parameters shown including clotting time (CT), clot formation time (CFT), maximum clot formation (MCF) and lysis parameters.

2.1.9 *In vitro* model of coagulopathy

A model of *in vitro* coagulopathy was designed based on the independent protocols of Caballo *et al* and Shenkman *et al* [315, 316]. Citrated whole blood was collected and diluted to 40% with 0.9% saline solution. Whole blood, diluted blood and blood treated 1:1 with PEP were then assessed by ROTEM. To better reproduce the coagulopathy associated with trauma, hypothermia was simulated in diluted blood, with or without PEP treatment, by measurement at 32°C [317]. Whole blood was measured at 37°C.

2.1.10 APTT, PT and fibrinogen

Activated partial thromboplastin time (APTT), Prothrombin time (PT) and Clauss fibrinogen assays were performed on a Sysmex CS-5100 coagulometer (Sysmex, Kobe, Japan) using Actin FS, Thromborel S and Dade® Thrombin Reagent (Siemens Healthineers, Marburg, Germany) on PEP, PPP and HPL.

2.1.11 Scott syndrome genetic sequencing

The recruited family with suspected Scott syndrome were sequenced using the R90 bleeding and platelet disorders targeted gene panel performed at Oxford Regional Genetics Laboratories and as previously described [318]. The final sequence variants were confirmed using Sanger sequencing.

2.1.12 Tissue factor antibody processing and conjugation

Human TF is a 263-amino-acid protein that comprises an extracellular domain (amino acids 1-219), a transmembrane domain (amino acids 220-242), and a cytoplasmic domain (amino acids 243-363) [319]. Several groups have generated monoclonal

antibodies against human TF [218, 320-324]. These antibodies can be broadly divided into 3 groups: group 1, antibodies that bind to the N-terminal region of the extracellular domain of TF and compete with FVII/FVIIa; group 2, antibodies that bind to the C-terminal region of the extracellular domain of TF that can bind to either free TF or TF complexed with FVII/FVIIa and interfere with substrate binding; group 3, antibodies that bind to the C-terminal region of the extracellular domain of TF close to the transmembrane membrane. In general, antibodies in group 2 are more potent inhibitors of the TF/FVIIa complex than antibodies in group 1, and antibodies in group 3 do not inhibit TF procoagulant activity [324-327]. I used HTF-1 to inhibit TF activity. This antibody has been used to inhibit TF activity in numerous studies and is commercially available [222, 320]. HTF-1 binds to a region of the N-terminal domain of the extracellular domain of TF that binds FVII/FVIIa [324, 325]. For the capture and detection of TF, I selected 1 antibody from group 2 and 1 antibody from group 1. The anti-TF antibody I selected from group 2 for the capture of TF was 5G9. 5G9 is commercially available (PABW-121, Creative Biolabs) that binds to the C-terminal domain of the extracellular domain of TF (amino acids 181-214, specifically amino acids 156, 169, 200, and 201) [325, 327]. The anti-TF antibody I selected from group 1 for detection of TF was IIID8. IIID8 is commercially available (4509, American Diagnostica) and binds to the N-terminal domain of the extracellular domain of TF (amino acids, 1-25) [325, 328]. I also used VIC12 in early experiments which is a group 3 antibody that binds to the N-terminal domain of the extracellular domain of TF (amino acids, 1-25) [329].

Anti-human TF antibodies 5G9 (a gift from Professor James Morrissey), IID8, VIC12 and HTF-1 (gifts from Professor Nigel. Mackman) [39] were obtained as unconjugated forms. The protein concentration of each antibody was confirmed upon receipt using the BCA protein assay (ThermoFisher, Massachusetts, US). Each antibody was further purified by immunoprecipitation using a bovine serum albumin (BSA) removal kit (AbCam, Cambridge, UK). Briefly, BSA removal buffer was incubated at 37°C for 10 minutes and was added to antibody at a ratio of 1:1.25, vortexed and incubated for 5 minutes at room temperature. Mixtures were centrifuged at 12,000 g for 5 minutes twice, to ensure pellet formation and the supernatant aspirated. The pellet was resuspended in 500 µl of 1 µm filtered phosphate buffered saline (PBS).

Ultra-0.5ml centrifugal filter units (Amicon, Harrogate, UK) were then used to concentrate antibodies. 500 µl of sample was added to the filter device placed inside filtrate collection tubes and centrifuged at 14,000 g for 30 minutes at room temperature. The filter device was removed, inverted and placed inside a new filtrate collection tube. Filter units were then centrifuged twice at 1000 g for 10 minutes, with the resultant liquid (15 µl) containing a highly concentrated, purified antibody.

Following measurement of total protein (BCA) in the purified antibody, TF antibodies were diluted to 1 mg/ml for conjugation to AF647 following manufacturer recommendations (Lightning-Link® Rapid Alexa Fluor® 647 Labelling Kit, Abcam). Briefly, 1 µl of modifier reagent was added to 10 µl of antibody, gently mixed and added to lyophilised AF647 and incubated in the dark at room temperature for 30 minutes. After incubation, 1 µl of quencher reagent was added and mixed gently. Conjugated

IID8-AF647 was diluted 1:5 to a total volume of 50 μ l. Sodium azide (Sigma Aldrich) was added at a final concentration of 0.01% and the preparation stored at 4°C.

To facilitate TF EV capture experiments, ExoFlex kits (Unchained Labs, Pleasanton, CA, USA) were used. 5G9 and HTF-1 anti-TF antibodies were diluted to 0.5 mg/ml. 8 μ l of ExoFlex modifier was added to 80 μ l of antibody and gently mixed. ExoFlex modifier-antibody mixture was then added to lyophilised ExoFlex Linkers. 5G9 was conjugated to ExoFlex Linker 1, HTF-1 was conjugated to ExoFlex linker 2 by incubation at room temperature for 45 minutes. 8 μ l of ExoFlex quencher was then added and incubated for 15 minutes at room temperature. Conjugated antibodies were stored at 4°C.

2.1.13 Growth Factor Characterisation by multiplex assay

Magnetic bead-based multiplex assays (Luminex) were used to assess the growth factor content (PDGF-AB, P-selectin, VEGF-alpha and CXCL7/NAP2) of PEP, PPP and HPL (R&D Systems, Minneapolis, USA). Briefly, 50 μ l of diluted samples or standards were added to 96 well plates. 50 μ l of analyte bead cocktail was added to each well and the plate was incubated for 2 hours at room temperature on a plate shaker at 800 rpm (LSE Digital Microplate Shaker, Corning). Following an automated programme, plates were washed three times with a magnetic plate washer. The antibody-biotin reporter was added and incubated for 1 hour at room temperature on a plate shaker at 800 rpm. Following three washes, 50 μ l of streptavidin-phycoerythrin was added to each well and plates were incubated at room temperature in the dark on a plate shaker for 30 minutes. Following a final three washes the plates were

measured on a Bioplex 100 plate reader (Bio-Rad Laboratories, Hercules, CA). 5-parameter logistic curves were generated from the standards.

2.1.14 BxPC-3 cell culture

BxPC-3 cells (ATCC, Virginia, USA) were thawed upon receipt for 10 minutes at 37°C and resuspended in complete growth medium (CGM) (RPMI 1640 supplemented with glutamine and penicillin/streptomycin, 10% Foetal Calf Serum (FCS)) for a final total volume of 12 ml split across two T25 flasks. CGM (10% FCS) was replaced every three days. Upon achieving confluence, cells were removed with trypsin and placed into T150 flasks.

For experiments, when T150 flasks reached 75% confluence they were washed with PBS and CGM was replaced with serum-free CGM. After three days of cell culture supernatants were collected. Conditioned media was centrifuged at 300 g for 5 minutes to remove large debris and subsequently double centrifuged at 2000 g for 20 minutes and 13,000 g for 2 minutes. Cell culture supernatant sample aliquots were then stored at -80°C.

2.1.15 Platelet flow cytometry

For platelet counting 20 µl of PFP was diluted 1:20 in 380 µl of filtered PBS containing 0.1% bovine serum albumin (PBS-BSA). 50 µL of diluted PFP was combined with 1 µl of anti-CD61-FITC (BD Biosciences, Oxford, UK) in standard 12 × 75-mm polystyrene tubes, incubated for 15 minutes at room temperature in the dark and diluted 1:5 to a total volume of 250 µl with PBS-BSA buffer, to achieve a final dilution factor of 1:100.

Samples were analysed at medium flow rate (<3000 events per second) on the Accuri™ C6, using an FSC-H threshold of 25,000 to exclude debris and electronic noise. Platelet flow cytometry direct volume counts were obtained by multiplying the displayed counts in the platelet gate (per μl) by the final dilution factor. For typical plasma samples the recommended final dilution factor is 1:1000 [330, 331]. For PFP and filtered PFP optimisation was required and a final dilution of 100 was instead used. For measurement of platelet activation 5 μl PRP was incubated for 15 minutes with 5 μl of the platelet degranulation/activation marker CD62p-FITC (Clone: CLBThromb/6, Beckman Coulter, California, US) in 40 μl of PBS-BSA with either 20 μM TRAP (thrombin receptor activating peptide) (gift from Professor Steve Watson) or 10 $\mu\text{g/ml}$ CRP (collagen related peptide) (gift from Professor Steve Watson) or 10 μM calcium ionomycin (Sigma-Aldrich, Massachusetts, US). After 15 minutes incubation at room temperature samples were diluted 1:50 in PBS-BSA for a final dilution of 1:1000 and measured by flow cytometry.

2.1.16 Monocyte flow cytometry

200 μl of unstimulated whole blood or LPS stimulated blood (5 hours, 10 $\mu\text{g/ml}$) was stained with 5 μl of CD14-FITC (clone TUK4; Dako, Cambridgeshire, UK) and 1 μl of either anti-TF antibody IIID8-AF6F7 (1:200), VIC12-AF647 (1:200) or 5G9-AF647 (1:200) for 20 minutes at room temperature. Stained whole blood was incubated for 20 minutes at room temperature. Following incubation 200 μl of whole blood was lysed with 2 ml of 1x lysis buffer (BD FACS lysis buffer, BD Biosciences, New Jersey, US) and incubated for 10 minutes in the dark. Following lysis, cells were centrifuged at 250

g for 5 minutes and resuspended in 200 μ l of PBS. Monocytes were gated on by their classical forward scatter and side scatter. Monocyte events (n = 2500) were then identified using CD14-FITC and anti-TF antibody conjugated to AF647 where median fluorescence intensity and % TF positive events were then measured (see Figure 2.3 for gating strategy).

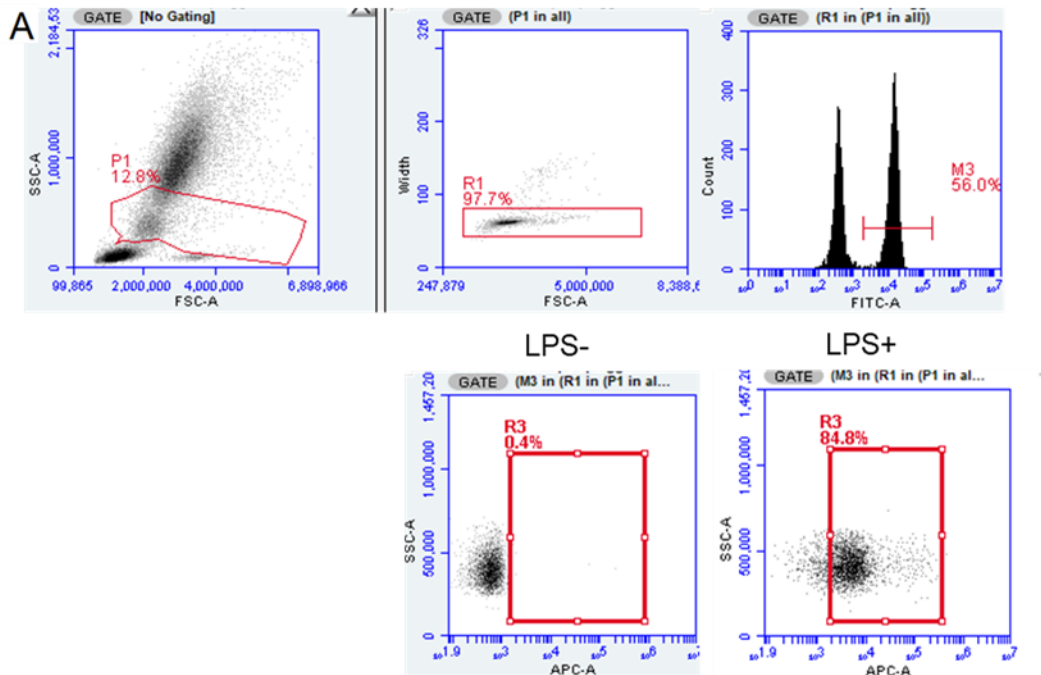


Figure 2.3. TF expression by LPS stimulated monocytes.

A) Flow cytometry gating strategy of monocytes (vehicle and LPS stimulated) from citrated whole blood. Monocytes were selected based upon forward scatter, side scatter and removal of doublets. From this gate, CD14-FITC+ positive monocyte events were selected. Anti-tissue factor (TF) antibody (IIID8-AF647) was used to identify TF positive events. Prior to labelling, blood was stimulated with 10 μ g/ml lipopolysaccharide for 5 hours at 37°C whilst being gently shaken.

2.1.17 Nanoparticle tracking analysis (NTA)

NTA was performed using a Nanosight NS300 instrument (Malvern Analytical Ltd, Malvern, UK) All samples were diluted in 0.22 μm filtered PBS to a final volume of 1 ml. Optimal measurement concentrations were determined by pre-testing the optimal particle per frame value (20–100 particles/frame). The following settings were set according to the manufacturer's software manual (NanoSight NS300 User Manual, AN0541-01-EN-00, 2017): camera level was increased until all particles were distinctly visible not exceeding a particle signal saturation over 20% (cell line-derived EVs: level 16). The optimal detection threshold was determined to include as many particles as possible with the restrictions that 10–100 red crosses were counted while only <10% were not associated with distinct particles. The blue cross count was limited to 5. Autofocus was adjusted so that indistinct particles were avoided. For each measurement, five 1-min videos were captured under the following conditions: cell temperature: 25°C; Syringe speed: 40 $\mu\text{l/s}$. After capture, the videos were analysed by NanoSight Software (NTA 3.1 Build 3.1.46 with a detection threshold of 8. Hardware: embedded laser: Blue405; camera: sCMOS). The number of completed tracks in NTA measurements was always greater than the proposed minimum of 1000 in order to minimise data skewing based on single large particles. Figure 2.4 shows NTA method and theory.

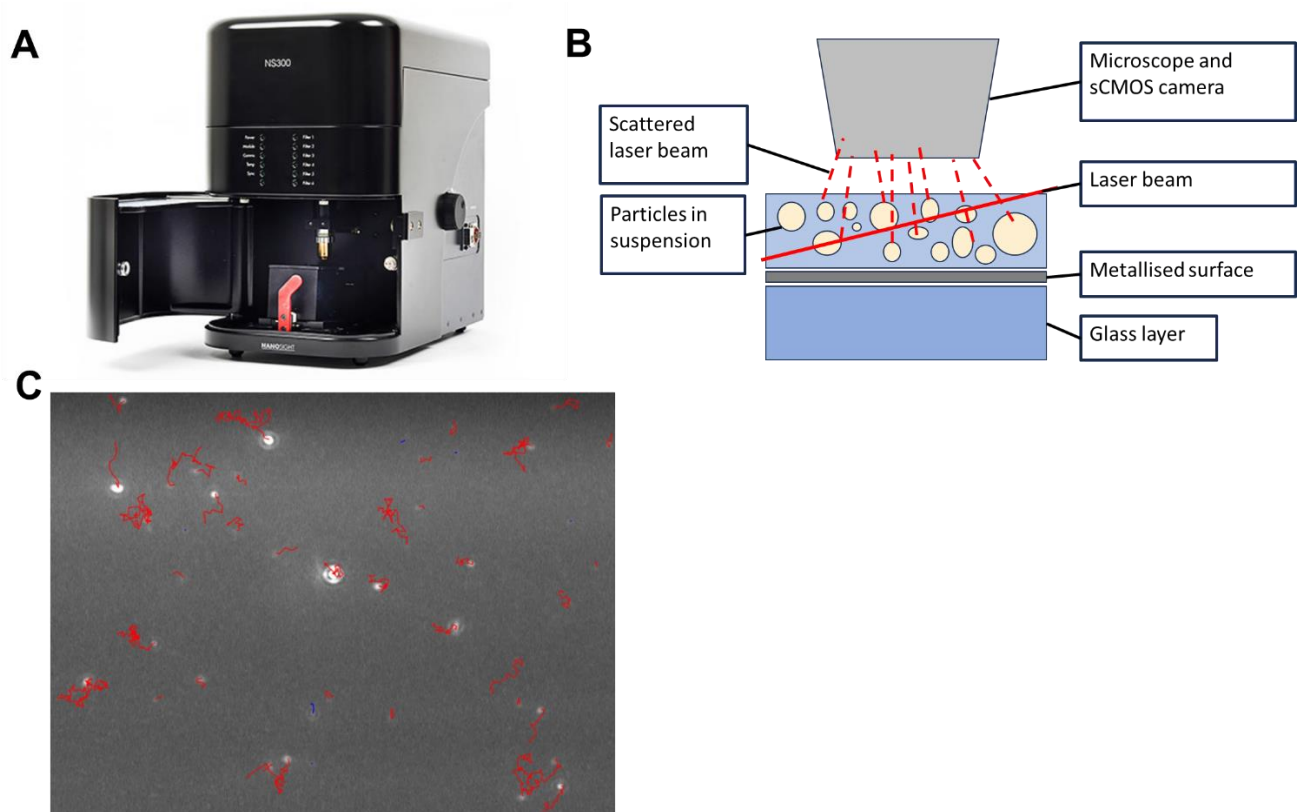


Figure 2.4. Nanoparticle tracking analysis (NTA) method and theory.

a) The NS300 instrument. b) Schematic showing particles in suspension and how laser light is scattered and received by a microscope and cMOS camera. c) A still image of video analysis showing how particles are tracked (in red), with blue dots indicating “noise” and events not considered particles based upon user-set detection threshold.

2.1.18 Measurement of extracellular vesicle tissue factor activity

Measurement of TF activity was conducted as previously described [20]. Briefly, 160 μ l of isolated EVs were required to measure samples in duplicate. Samples were divided into two aliquots of 80 μ l. To one aliquot, 22 μ l of inhibitory mouse anti-human TF (clone: HTF-1, BD Biosciences, San Jose, CA) (final concentration: 7.8 μ g/mL) was added. To the second aliquot 22 μ l of control mouse IgG (Sigma Aldrich) was added. Re-lipidated recombinant TF (Dade Innovin, Siemens, Munich, Germany) was used as a TF standard (0, 0.32, 0.63, 1.25, 2.5, 5, 10, 20 pg/ml). 50 μ l of TF standard or 40 μ l of sample was added, in duplicate, to the appropriate wells of a 96-well plate and incubated at room temperature for 15 minutes. 50 μ l of a mixture of human FVIIa (4.8 nmol/L stock, Enzyme Research Laboratories, South Bend, IN, USA) at a final concentration: 2.4 nmol/L and FX (146.4 nmol/L stock, Enzyme Research Laboratories) at a final concentration of 73.2 nmol/L in HEPES buffer saline with bovine serum albumin (HBSA) with CaCl_2 (10 mM/L) at a final concentration of 5 mmol/L added to each well. Plates were sealed with adhesive plate sealing film and incubated at 37°C for 2 hours. FXa generation was stopped by the addition of 25 μ L of HBSA with EDTA (25 mmol/L) at a final concentration of 5 mmol/L] to each well. 25 μ l of chromogenic substrate (Pefachrome FXa 8595, 4 mmol/L stock, Enzyme Research laboratories) at a final concentration of 0.67 mmol/L in water was added to each well and incubated at 37°C for 15 minutes. Absorbance at 405nm was then measured using a plate reader (BioTek EL808). Conversion of raw data to TF activity was performed following the formula: a: TF-dependent FXa generation (EV TF activity

[pg/mL]) = Total FXa generation (control IgG well) – TF independent FXa generation (HTF-1 well).

2.1.19 EV phenotyping with ExoView

EV were detected by immunocapture on chips using the ExoView R100 reader (Unchained Labs, Pleasanton, CA, USA) [332]. For different experiments different chip types were used, including Tetraspanin plasma chips (Unchained Labs, Pleasanton, CA, USA) and ExoFlex chips (Unchained Labs, Pleasanton, CA, USA). Details and schematic representation of ExoView R100 and chips are shown in Figure 1.9 under section titled “1.5.13 Immunoaffinity”. Tetraspanin plasma chips were arrayed with capture antibodies against human CD63, CD81, CD9, CD41a and Mouse IgG. ExoFlex chips were arrayed with the same capture antibodies as tetraspanin plasma chips with the addition of peptide material “ExoFlex 1” and “ExoFlex 2” allowing for customised antibody capture. Multiple protocols were used for ExoView work, including the tetraspanin plasma kit protocol, the ExoFlex kit protocol and an in-house generated protocol to allow for annexin-V staining. Details of the different protocols are shown in the subsections entitled “Tetraspanin Plasma Chip protocol”, “In-house annexin V protocol” and “ExoFlex Chip Protocol”. Before detailing the different protocols for ExoView I have first outlined the basic principles of using ExoView chips below.

2.1.17.1 Chip Pre-Scan

A basic overview of ExoView protocol is shown in Figure 2.5. Prior to use chips were pre-scanned to account for any background either from particles or to measure the inherent light scattering properties of the printed antibodies. Special care must be taken handling chips so as to ensure no damage occurs to the chip, in practice this involves using carbon-fibre tipped tweezers and only touching the edge of the chips with them (i.e. not the central square where antibody spots are printed).

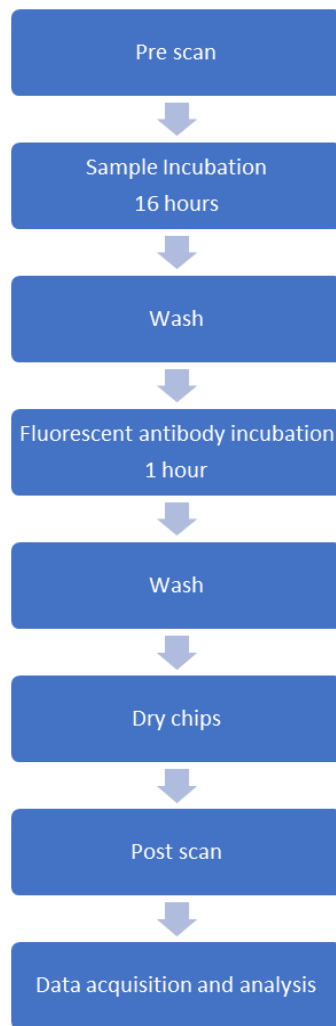


Figure 2.5. Overview of ExoView protocol.

ExoView protocol starts with the pre-scanning of chips pre-arrayed with arrayed with capture antibodies against target EV surface markers. After the pre scan, samples are incubated on the surface of the chip for 16 hours. After sample incubation chips are washed three times for three minutes each time in Solution A to remove any unbound material. After washing a panel of desired fluorescent antibodies are then incubated for 1 hour. Chips are then washed again to remove any unbound fluorescent antibody. After the final wash steps chips can be gently dried through careful exit from water using tweezers. Chips can then be post scanned and data acquired and analysed.

2.1.17.2 Sample incubation

35 µl of a pre-diluted sample were added to the surface of each chip. It is worth noting that each chip is single use and can only measure one sample. Each chip has antibody capture spots printed in triplicate. Each spot acts as an independent test interacting with only a small volume of sample. Samples are left to incubate on the surface of the chip for 16 hours overnight. The EV will settle onto the chip and bind to capture antibody if they express the antigen of interest. This effectively acts as an EV purification step potentially allowing for use of complex biofluids with no pre-isolation requirements. Once chips have sample loaded on their surface it is important that they are not allowed to dry prematurely. To aid this, chips are placed in the centre of a well within a 24 well plate, the void space between wells contains 1 ml of distilled water. Upon sealing with adhesive tape, the 24-well plate effectively becomes a moisture chamber that impedes evaporation of sample. After the 16-hour incubation is complete the chips are washed to remove unbound EV and primary antibodies conjugated to fluorophore can then be added to specify co-localised markers. Following fluorophore-conjugated antibody incubation it is imperative that unbound antibody be washed off and then begins the final stages of chip handling: drying.

2.1.17.3 Drying chips

After sample incubation and fluorophore-conjugated antibody labelling chips enter a 10ml petri dish filled with distilled water. The drying process is delicate and begins with deliberate and purposeful agitation of the chips through use of the tweezers. This is then followed by slow removal at a 45° angle from the petri dish. The surface tension of the distilled water should “pull” liquid off the chip. This is visible to the naked eye and

chip drying should be restarted if this does not occur. Improper drying may lead to unreliable results.

2.1.17.4 Post Scan

Following post scan measurement, ExoView Analyser software allows for the subtraction of pre scan data from the post scan data.

2.1.17.5 Data acquisition

The data that originates from a pre scan and a post scan can be exported through use of ExoView Analyser software. Two major types of data are generated with the first being interferometric imaging at 410nm excitation wavelength. This allows for enumeration and sizing of particles >50nm that are present on the surface of the chip bound to each antibody capture spot. The second type of data comes from fluorescent imaging. Fluorescence imaging allows for assessment of co-localised vesicle markers with more specific visualisation. Two direct capture and detection antibodies (surface and fluorophore conjugated antibody) are required for positive signal. Fluorescent imaging cannot determine EV size. It is worth noting that the particle count obtained from ExoView Analyser is termed “normalised particles”. This count is normalised to the size of the capture spot and the surrounding volume it interacts with. As shown in Figure 1.10 the zone of interaction between sample and antibody capture spots are discrete and non-overlapping.

This normalised particle count can then be converted into particles/ml by multiplying by the dilution factor and the constant: 1×10^4 (determined by spot size and interacting volume). The linear range of the ExoView R100 are counts between 10-5500

normalised particles with an estimated capture efficiency of over 95%. It worth noting that values above 5500 normalised particles are reaching saturation, this was tested in a series of preliminary experiments, where linearity of sample dilution was only observed between 10 and 5500 normalised particles (data not shown).

The earliest iteration (versions) of this software only allowed for basic outputs including normalised particle counts in interferometric and fluorescent mode as well as size (50-200nm) in interferometric mode. Within the ExoView analyser software a QC process is available which flags spots with warnings if they have a high count, or a high coefficient of variation (CV). Spots with warnings can be viewed and the operator can determine whether the spot is problematic and whether it should be removed from analysis. Problematic spots will typically be scratched and appear in light scatter mode with thick black lines on their surface, these are normally caused by scratching with tweezer during drying.

Following QC, the next step is thresholding the fluorescent data. Thresholding is an important step that acts to define the background fluorescence. Thresholding occurs on a chip-by-chip basis allowing any background signal to be removed from each antibody capture spot based on the fluorescent signal present on control Mouse IgG capture spots. Having completed QC, the final steps of data acquisition are to export the corrected data. A standardised approach to thresholding was used setting the Mouse IgG fluorescent normalised particle count to 10. Figure 2.6 shows a typical representative image of a fluorescent image that ExoView can generate.

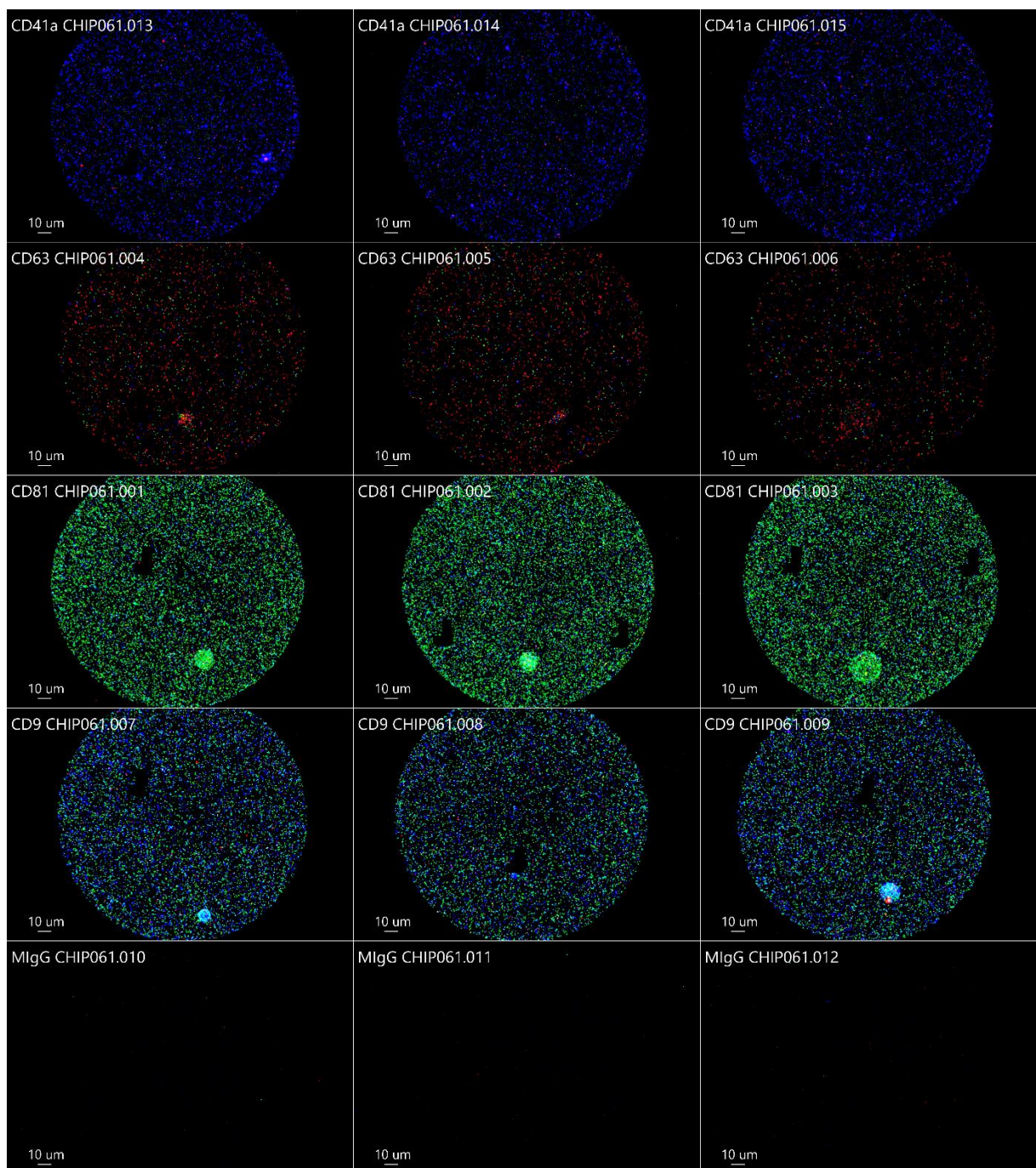


Figure 2.6. Example fluorescent images of a one sample on one chip.

Individual small round “dots” are the EV that have been captured by antibody against either CD41, CD63, CD81, CD9 or Mouse IgG. Captured EV were fluorescently labelled with three fluorescent antibodies (CD9-AF488 in blue, CD63-AF647 in red and CD81-AF555 in green). This sample was PFP diluted to 1:10 prior to incubation with the chip.

2.1.17.6 Tetraspanin Plasma Chip protocol

The tetraspanin plasma protocol followed the kit provided protocol. 35 μ l of dilute sample was applied to each chip. Distilled water was added to the void space between wells of a 24 well plate, the plate was sealed and incubated for 16 hours at room temperature in the dark. The 16-hour incubation then ended with the addition of 1 ml of Solution A. The plate containing chips was then shaken at 500 rpm (LSE Digital Microplate Shaker, Corning, New York, USA) for three minutes. 750 μ l of Solution A was removed from each well. This left 250 μ l of solution remaining, this was to ensure chips did not prematurely dry. Chips were washed by adding 750 μ l, shaking for three minutes with subsequent replacement of 750 μ l of Solution A. This was repeated for a total of three times. Following the final wash, 750 μ l was removed from each well. To the 250 μ l remaining in each well 250 μ l of antibody cocktail diluted in kit-provided blocking solution was applied to each chip. Details of antibodies used, their source and dilutions are shown in Table 2.1. It is also worth noting plasma samples were ran with isotype controls at the beginning of my PhD, mouse IgG2b was found to exhibit non-specific binding but IgG1, IgG2a and IgM all appear suitable (data not shown). Chips were then incubated for one hour at room temperature in the dark being shaken at 500 rpm. After this incubation 500 μ l of Solution A was added to each well. 750 μ l was then removed and replaced with 750 μ l of Solution A. Plates were shaken at 500 rpm for three minutes. Following this 750 μ l was removed from each well and replaced with Solution B and shaken at 500 rpm for 5 minutes. Solution B replacement and shaking was repeated for a total of three times. Then a final 750 μ l replacement was made with distilled water. Chips were then moved to 10 cm diameter petri dishes filled

with 20 ml of distilled water. Using carbon-fibre tipped tweezers, chips were carefully washed in petri dishes and then slowly removed at a 45° angle ensuring that any liquid on chips was removed through surface tension forces. Solution A and B are proprietary buffers but are likely to be PBS for solution A and PBS and tween for solution B.

2.1.17.7 In-house annexin V protocol

The in-house protocol annexin-V protocol involved diluting samples in HEPES buffered saline (HBS). 35 µl of diluted sample was applied to each chip. Distilled water was added to the void space between wells of a 24 well plate, the plate was sealed and incubated for 16 hours at room temperature in the dark. Chips were washed three times with HBS supplemented with 2.5 mM Ca²⁺ and 15 µM GPRP (Gly-Pro-Arg-Pro-amide) (Sigma Aldrich). Following the final wash, 750 µl was removed from each well. To the 250 µl remaining in each well 250 µl of antibody cocktail diluted in 4% BSA solution and supplemented with 15 µM GPRP amide was added to each well. After each wash the plate was shaken at 500 rpm (LSE Digital Microplate Shaker, Corning) for three minutes. Following the final wash, 750 µl was removed from each well. To the 250 µl remaining in each well 250 µl of antibody cocktail diluted in kit-provided blocking solution was applied to each chip. Chips were then incubated for one hour at room temperature in the dark being shaken at 500 rpm. After this incubation 500 µl of with HBS supplemented with 2.5 mM Ca²⁺ were added and incubated for one hour at room temperature in the dark. Chips were washed three times as previously. A final wash was performed with distilled water. Chips were then moved to 10 cm diameter petri dishes filled with 20 ml of distilled water. Using carbon-fibre tipped tweezers,

chips were carefully washed in petri dishes and then slowly removed at a 45° angle ensuring that any liquid on chips was removed through surface tension forces. The chips were imaged with the ExoView R100 reader (nScan2 Version 2.76 software). The data was analysed using Nano Viewer Version 2.82 with fluorescence gating based on Mouse IgG capture background and sizing thresholds set at 50 nm to 200 nm.

2.1.17.8 ExoFlex Chip Protocol

The ExoFlex chip protocol followed the kit provided protocol. ExoFlex chips allow for the addition of two custom capture antibodies via linker conjugation to peptide material through the printing of peptide material labelled “ExoFlex 1” and “ExoFlex 2”. ExoFlex 1 and ExoFlex 2 bind to Linker 1 and Linker 2, respectively. The details of antibody processing and conjugation to Linker 1 and 2 are described above under the heading “Tissue factor antibody processing and conjugation”. To allow binding of conjugated antibody to “ExoFlex 1” and “ExoFlex 2”, linker-conjugated antibodies were allowed to equilibrate to room temperature and were then diluted 1:100 in solution A and 35 µl applied to each chip for 30 minutes. To remove any unbound material and prepare chips for sample incubation they were washed following manufacturer recommendation with kit provided solutions. Briefly, 1 ml of Solution A (Unchained Labs, Pleasanton, CA, USA) was added to each well. 750 µl of solution within wells was then discarded. 750 µl of Solution B (Unchained Labs, Pleasanton, CA, USA) was added to each well. 750 µl of solution within wells was then discarded and replaced with 750 µl of 1 µm filtered distilled water. Chips were carefully removed and placed in petri dishes (10 cm diameter) containing 1 µm filtered distilled water. Chips

were washed and dried and then placed in separate wells of a new 24-well plate. Protocol then followed the standard tetraspanin plasma protocol as detailed above.

Table 2.1. Antibody details for ExoView experiments.

Antibody/Marker	Fluorophore	Clone	Isotype	Source	Dilution	Chip Type
CD81	AF-555	M38	IgG1	Unchained Laboratories	1:500	Tetraspanin plasma
CD63	AF-647	Ts63	IgG1	Unchained Laboratories	1:500	Tetraspanin plasma
CD9	AF-488	Ts9	IgG1	Unchained Laboratories	1:500	Tetraspanin plasma
CD63	PE	H5C6	IgG1	ThermoFisher	1:1000	ExoFlex
CD41a	PE	MWReg30	IgG1	Biorbyt	1:1000	Tetraspanin plasma
CD14	FITC	TUK4	IgG2a	Dako	1:250	ExoFlex
Tissue Factor	AF-647	IIID8	IgG1	American Diagnostics	1:500	ExoFlex
GPVI	PE	HY101	IgG1	BD Pharmingen	1:200	Tetraspanin plasma
CLEC1B	APC	AYP1	IgG1	Biolegend	1:200	Tetraspanin plasma
LAMP1/CD107A	FITC	eBioH4A3	IgG1	Thermofisher	1:83	Tetraspanin plasma

Annexin V/PS	FITC	N/A	N/A	BD Pharmingen	1:200	Tetraspanin plasma
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2.2 Statistical Analysis

Statistical analysis and figures were generated using RStudio [333]. For all datasets where more than two groups are being compared Kruskal-Wallis tests were performed followed by Wilcoxon signed rank tests where sample groups were related and Wilcoxon rank sum tests where samples groups were unrelated. The Holm-Bonferroni method was applied to correct for multiple comparisons. Where datasets contained two samples the Wilcoxon signed rank test was used to determine significance. Values reported in text are median values. The predefined level of significance was set at 5% (alpha level = 0.05). Non-parametric statistics were chosen because the relatively small n used for experiments which precludes accurate normality calculation and the broad variances within experimental groups which is another key assumption of parametric tests. It is also worth noting that the small n of experiments may be leading to inadequate power to determine if an effect is or is not observed.

Chapter 3. Platelet Enhanced Plasma

Some of this work has been published under the title: "Platelet-enhanced plasma: characterization of a novel candidate resuscitation fluid's extracellular vesicle content, clotting parameters, and thrombin generation capacity".

Price *et al*, 2021. DOI: [10.1111/trf.16423](https://doi.org/10.1111/trf.16423)

My contribution to this publication included writing the manuscript and performing the vast majority of the experiments. Dr Chris Gardiner conducted APTT, PT and fibrinogen tests but all other data and experiments were designed and carried out by me.

Additionally, some parts of this work are under review under the title "Comprehensive functional characterisation of a novel ANO6 variant in a new patient with Scott syndrome" (*in press*).

My contribution to this publication included the EV measurements, genetic sequencing and organising family pedigree was performed by Dr Samantha Montague.

3.1 Rationale

Platelets exhibit a relatively short shelf life for transfusion purposes of approximately 6 days. They also require stringent storage conditions with constant gentle agitation required at room temperature to maintain their function. Despite this the platelet storage lesion still occurs with a loss of platelet functionality during storage. All of this limits their usefulness in a clinical setting and significantly contributes to wastage with disposal of expired concentrates. Plasma on the other hand is more easily stored long-term. A fluid similar to HPL (human platelet lysate) could combine the benefits of platelets and plasma in one formulation that is more easily stored and reliant, partially on, the procoagulant EV profile of PEV released upon freeze-thaw cycling. In addition to assessing PEP as a potential resuscitation fluid it may also provide an ideal sample type to test ExoView and develop a protocol for measurement of PS-expressing EV without requiring EV isolation methodologies. In addition to assessing PEP as a potential novel resuscitation fluid it also provides an opportunity to test whether

ExoView can detect PS-positive platelet derived EV and to compare with other procoagulant tests.

3.2 Aims

- To generate platelet-enhanced plasma (PEP) and assess its functionality via clotting tests and thrombin generation dynamics.
- To measure EV in PEP using ExoView.
- To develop a protocol for measurement of PS-expressing EV on ExoView for application to PEP and Scott syndrome.
- To assess the *in vitro* coagulant properties of PEP in a model of dilutional coagulopathy.
- To compare PEP to existing commercially generated HPLs.

3.3 Results:

3.3.1 Blood Cell Count

PEP was generated by 3 cycles of freeze-thawing PRP followed by centrifugation to remove platelet remnants. Each step of blood processing underwent whole blood cell counting (Sysmex XN1000) including whole blood, PRP, PFP, PPP and PEP. A summary of cell counts in whole blood, PPP, PRP, PEP and PFP (n = 6) are shown in Figure 3.1. The platelet count of whole blood ($252 \times 10^9/L$, $p = 0.031$) was less than PRP ($376 \times 10^9/L$), greater than PPP ($9.5 \times 10^9/L$, $p = 0.031$), PEP ($6 \times 10^9/L$, $p = 0.031$) and PFP ($<1 \times 10^9/L$, $p = 0.031$). The erythrocyte counts in whole blood ($4.94 \times 10^{12}/L$) were greater than PRP ($0.03 \times 10^{12}/L$, $p = 0.035$), PPP ($0.00 \times 10^{12}/L$, $p = 0.036$), PEP ($0.00 \times 10^{12}/L$, $p = 0.031$) and PFP ($0.01 \times 10^{12}/L$, $p = 0.036$). As expected, the number

of leukocytes was lower in PRP ($0.01 \times 10^9/\text{L}$, $p = 0.031$), PPP ($0.00 \times 10^9/\text{L}$, $p = 0.031$), PEP ($0.00 \times 10^9/\text{L}$, $p = 0.031$) and PFP ($0.00 \times 10^9/\text{L}$, $p = 0.031$) compared to whole blood ($6.63 \times 10^9/\text{L}$). Freeze-thaw cycling of PRP and centrifugation generates PEP which is characterised by a significantly reduced platelet count ($p = 0.031$) and is free of leukocyte and erythrocyte contamination.

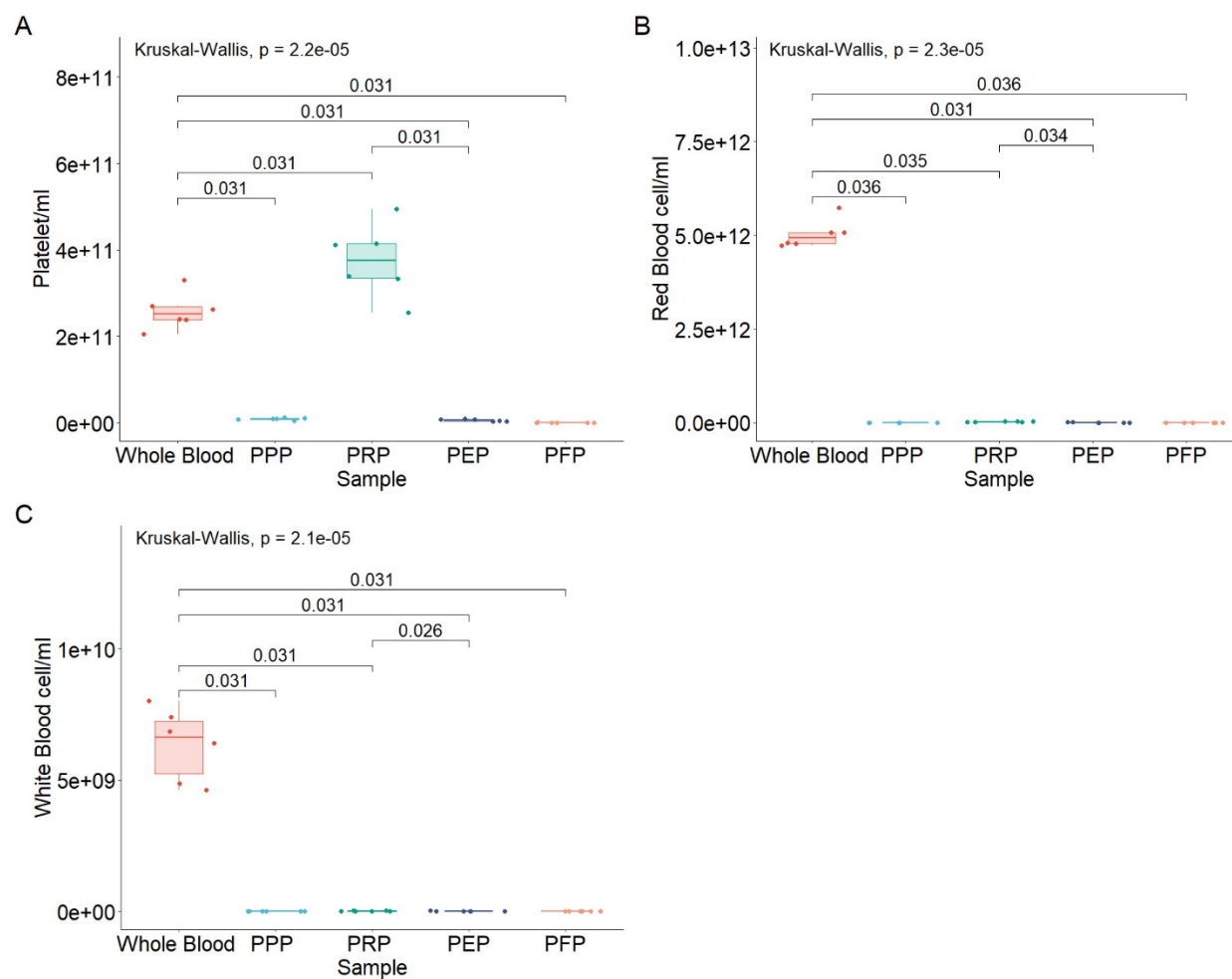


Figure 3.1. Cell counts in whole blood, PPP, PRP, PEP and PFP.

a) Platelet counts b) Erythrocyte counts c) Leukocyte counts ($n = 6$). Kruskal-Wallis tests followed by Wilcoxon sign rank tests, with selected significant differences shown.

3.3.2 PEP Clotting Characteristics

Having shown PEP to be acellular I next sought to determine the clotting characteristics of PEP against PPP with comparison to fresh (i.e. not freeze-thawed) PRP and PPP. Figure 3.2a shows that PEP (41s) exhibits an enhanced CT with EXTEM stimulation compared to fresh PPP (51s, $p = 0.021$) and PRP (51s, $p = 0.012$). No differences were observed in INTEM CT between groups (Figure 3.3a). With EXTEM and INTEM testing there were no significant differences in CFT between groups (Figure 3.2b and 3.3b). MCF (Figure 3.2c) is significantly greater in PRP (79 mm) with EXTEM testing compared to PPP (24mm, $p = 0.0079$), and PEP (27mm, $p = 0.0079$). With INTEM testing, MCF is also greater in PRP (79mm) compared to PPP (23mm, $p = 0.012$) and PEP (26mm $p = 0.0079$). With EXTEM and INTEM testing alpha angle was not significantly different amongst groups (Figure 3.2d, Figure 3.3d). No significant differences in APTT, PT or fibrinogen were observed between PEP and PPP (Figure 3.4). The key takeaway of ROTEM characterisation is CT is rapid in PEP.

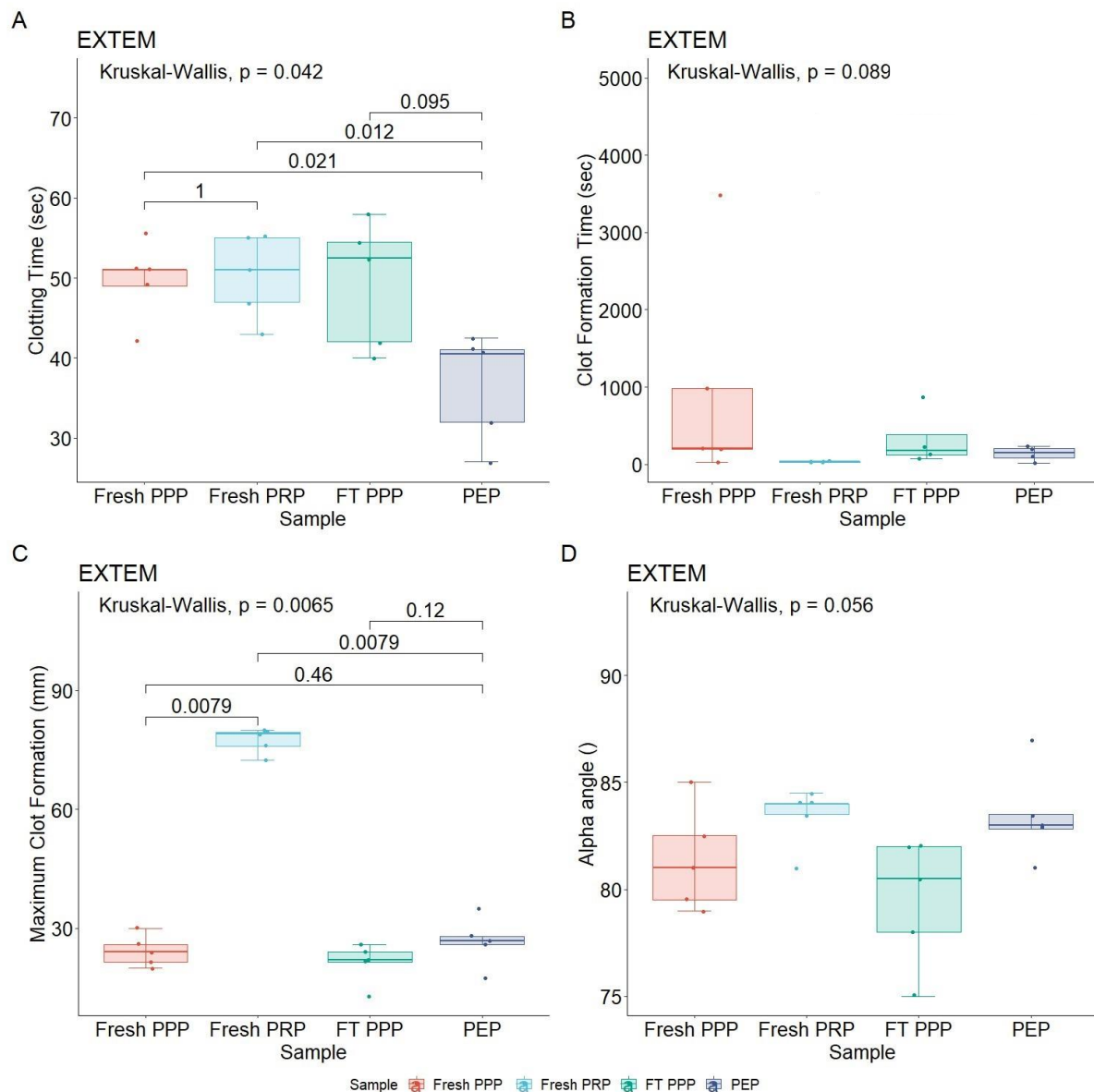


Figure 3.2. EXTEM Clotting parameters of PPP (fresh and freeze-thawed), PRP and PEP measured by ROTEM.

a) EXTEM Clotting time, b) EXTEM Clot Formation Time, c) EXTEM Maximum Clot Formation, d) EXTEM Alpha Angle). Kruskal-Wallis tests followed by Wilcoxon sign rank tests. ($n = 5$, measured in duplicate).

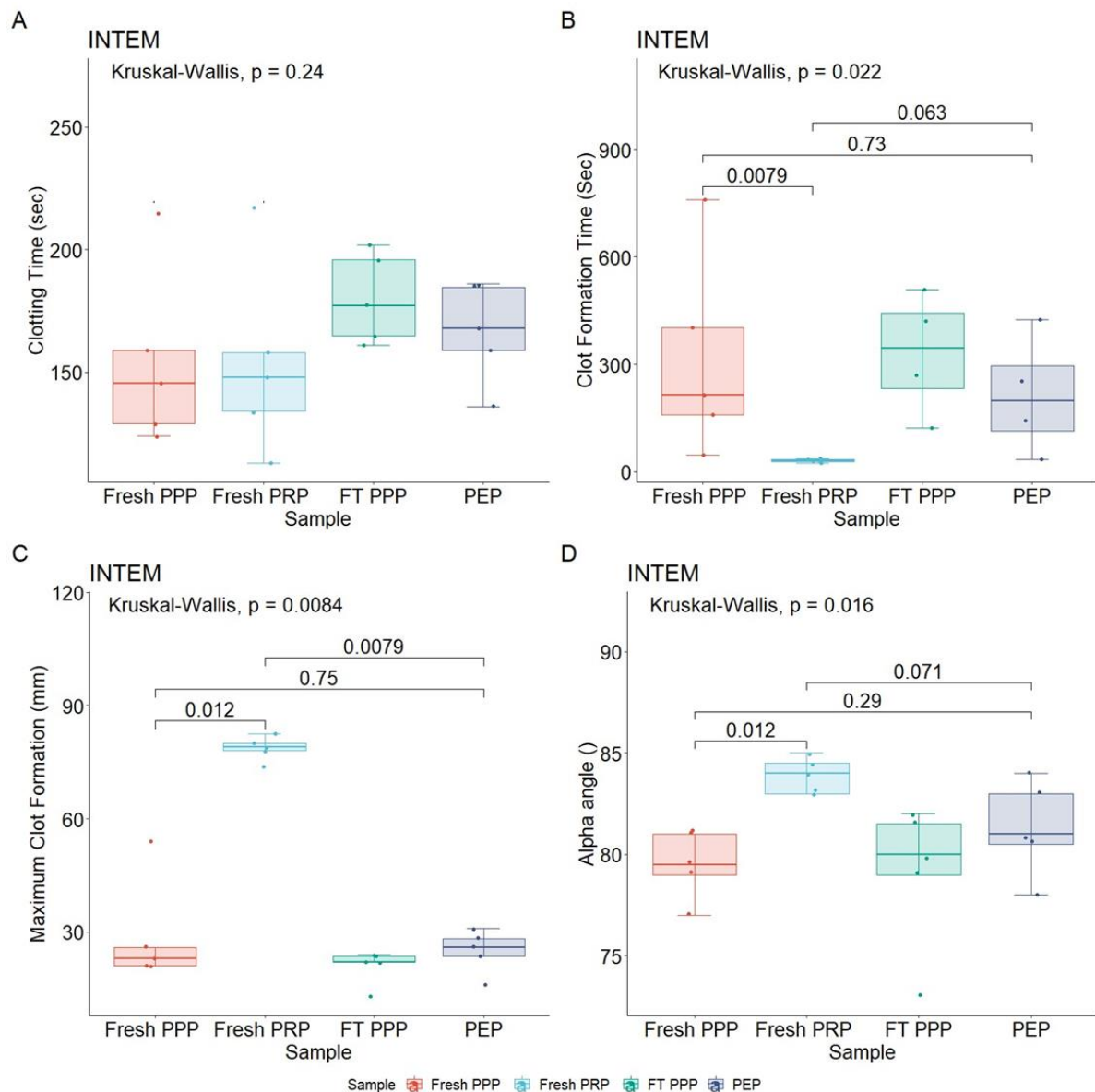


Figure 3.3. INTEM Clotting parameters of PPP (fresh and freeze-thawed), PRP and PEP measured by ROTEM.

a) INTEM Clotting time, b) INTEM Clot Formation Time, c) INTEM Maximum Clot Formation, d) INTEM Alpha Angle). Kruskal-Wallis tests followed by Wilcoxon sign rank tests. ($n = 5$, measured in duplicate).

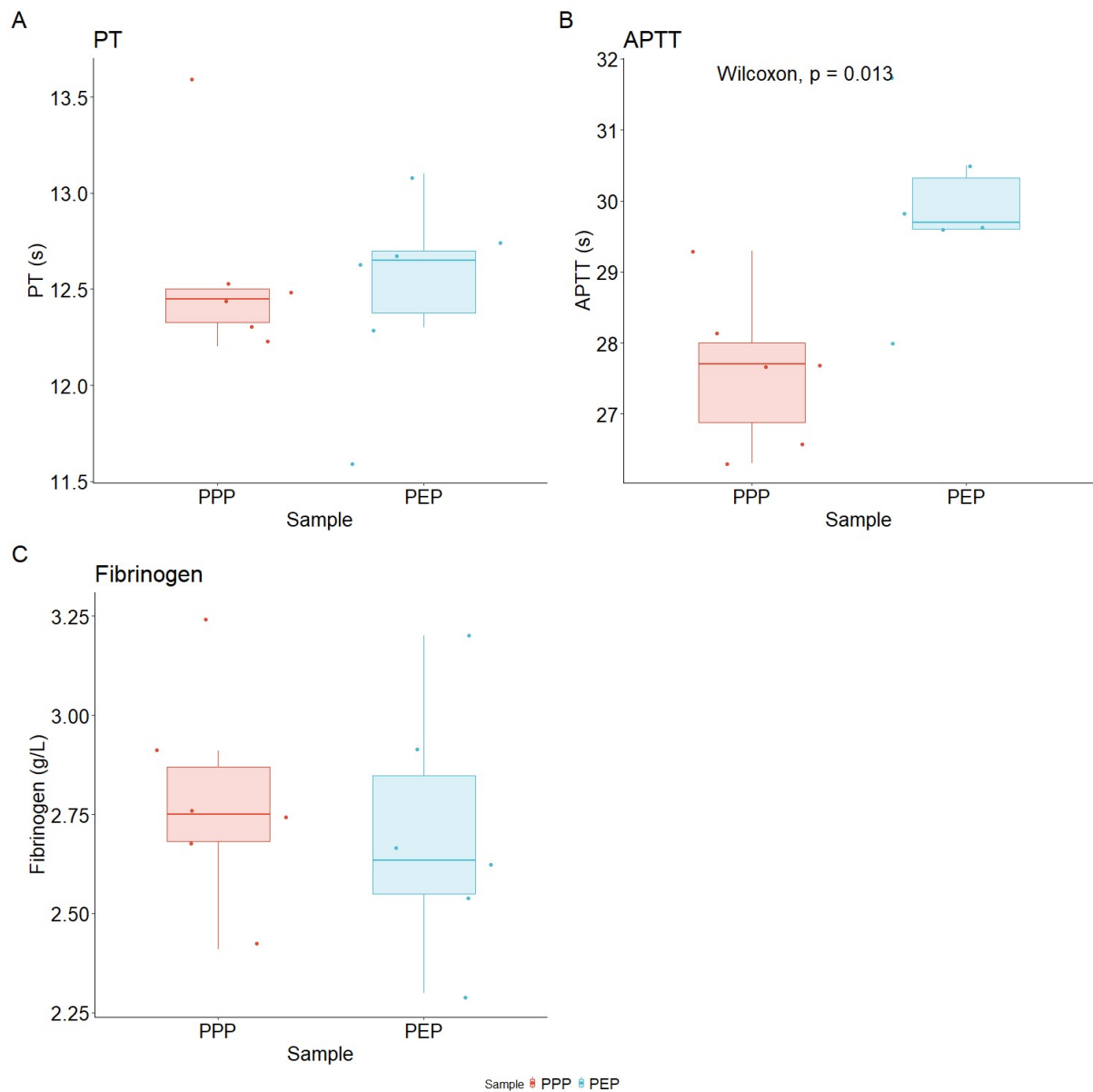


Figure 3.4. Clotting parameters of PPP (fresh and freeze-thawed), PRP and PEP measured by Sysmex CS5100.

a) Sysmex CS5100 PT, b) Sysmex CS5100 APTT, c) Sysmex CS5100 Fibrinogen. Kruskal-Wallis tests followed by Wilcoxon sign rank tests. ($n = 6$, measured in duplicate). Data for this figure and experiment were performed by Chris Gardiner.

3.3.3 PEP Treatment of an In Vitro Model of Coagulopathy: Clotting Characteristics

To test whether PEP could successfully treat coagulopathic blood I generated an *in vitro* model based on two separate protocols that together simulate dilutional coagulopathy and hypothermia. With EXTEM the CT of the dilutional model of coagulopathy (122 s) was increased compared to whole blood (82 s, $p = 0.012$) and PEP treatment of the coagulopathic blood (56 s) resulted in a shorter CT compared to both whole blood ($p = 0.012$) and the model ($p = 0.012$) (Figure 3.5a). CT of the dilutional model (302 s) was increased with INTEM testing compared to whole blood (159 s, $p = 0.012$) and restored upon PEP treatment (151s, $p = 0.016$), with no significant difference to whole blood CT ($p = 0.53$) (Figure 3.6a). CFT with EXTEM testing was increased in the coagulopathic blood (257s) compared to whole blood (62s, $p = 0.0079$) and was not restored upon PEP treatment (170 s, $p = 0.15$) remaining significantly longer than whole blood CFT ($p = 0.0079$) (Figure 3.5b). CFT with INTEM followed a similar pattern with the model successfully increasing CFT (266s, $p = 0.0079$) compared to whole blood (58s), but PEP did not rescue this (183s, $p = 0.42$) (Figure 3.6b). The MCF of whole blood (67 mm) with EXTEM testing was reduced in the model conditions (43 mm, $p = 0.0079$) and did not significantly increase with PEP treatment (50 mm) ($p = 0.25$) (Figure 3.5c). INTEM testing shows that MCF is reduced in the model (45 mm) compared to whole blood (67 mm, $p = 0.012$) and is not significantly different upon PEP treatment (44 mm, $p = 0.75$) which remained lower than whole blood MCF ($p = 0.012$) (Figure 3.6c). With EXTEM testing the alpha fell in the model conditions (49) compared to whole blood (75, $p = 0.0079$) (Figure 3.5d). Upon PEP treatment EXTEM alpha (78, $p = 0.012$) was significantly improved and

similar to whole blood alpha (75, $p = 0.75$) (Figure 3.5d). With INTEM testing model alpha (47) was lower than whole blood alpha (78, $p = 0.012$), PEP treatment improved alpha (79, $p = 0.012$), restoring to a similar value to whole blood ($p = 0.67$) (Figure 3.6d). Taken together these results show that PEP contains a similar concentration of fibrinogen to PPP which was expected. PEP successfully reduces CT but does not restore MCF.

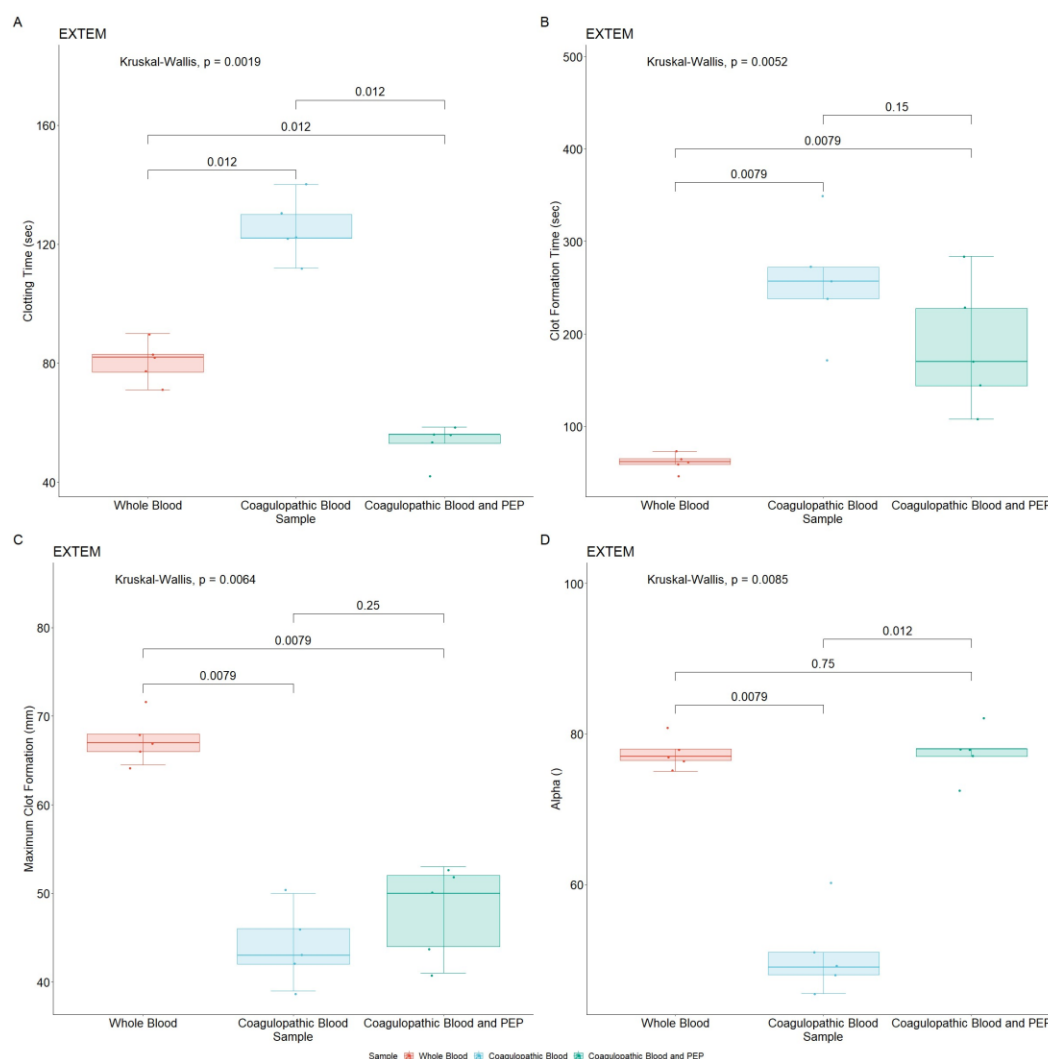


Figure 3.5. EXTEM clotting parameters of whole blood, a dilutional coagulopathy model (Whole blood diluted to 40% with saline and measured at 32°C) and PEP treatment of the coagulopathy model measured by ROTEM.

a) EXTEM Clotting time, b) EXTEM Clot Formation Time, c) EXTEM Maximum Clot Formation, d) EXTEM Alpha Angle. Kruskal-Wallis tests followed by Wilcoxon sign rank tests. $n = 5$, measured in duplicate.

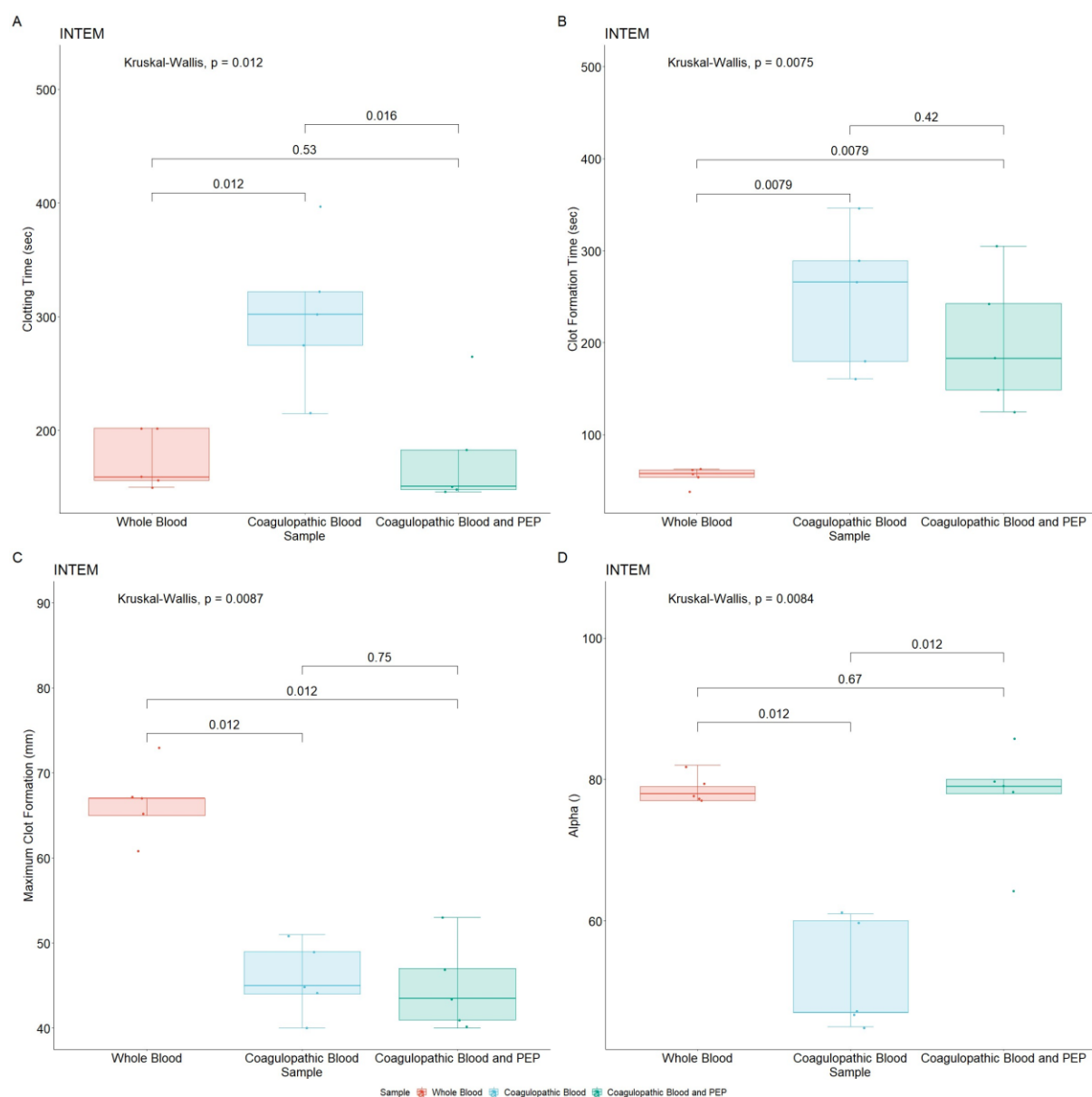


Figure 3.6. INTEM clotting parameters of whole blood, a dilutional coagulopathy model (Whole blood diluted to 40% with saline and measured at 32°C) and PEP treatment of the coagulopathy model measured by ROTEM.

a) INTEM Clotting time, b) INTEM Clot Formation Time, c) INTEM Maximum Clot Formation, d) INTEM Alpha Angle. Kruskal-Wallis tests followed by Wilcoxon sign rank tests. $n = 5$, measured in duplicate.

3.3.4 Commercial Lysate Clotting is Impaired but Rescued with Fibrinogen Supplementation

HPLs exhibit impaired clotting, in some cases no clot could be detected by use of Sysmex Coagulometer (Table 3.1). Upon exogenous addition of fibrinogen to 2mg/ml, 3 mg/ml and 4 mg/ml the clotting time increased in a dose-dependent manner with increasing fibrinogen supplementation across all three commercial lysates, CFT was robustly reduced (Figure 3.7 and 3.8). MCF increased with increasing fibrinogen supplementation.

All three commercial lysates did not form a clot with ROTEM testing with EXTEM or INTEM reagent prior to fibrinogen supplementation (Table 3.1). Sysmex coagulometer data shows Trinova Biochem platelet lysate did not form a clot. But interestingly MacoPharma platelet lysate and PL Bioscience commercial lysate did form clots. The fibrinogen concentration in all commercial lysates was below 0.8 g/L which probably contributed to their abnormal clotting.

Table 3.1. Clotting characteristic of commercial lysates as measured by sysmex coagulometer and ROTEM.

NCD = No clot detected

Sample	PT (s)	APTT (s)	Fibrinogen (g/L)	EXTEM	INTEM
MacoPharma	20.4	58.2	0.79	NCD	NCD
Trinova Biochem	NCD	NCD	NCD	NCD	NCD
PL Bioscience	32	69.5	0.38	NCD	NCD

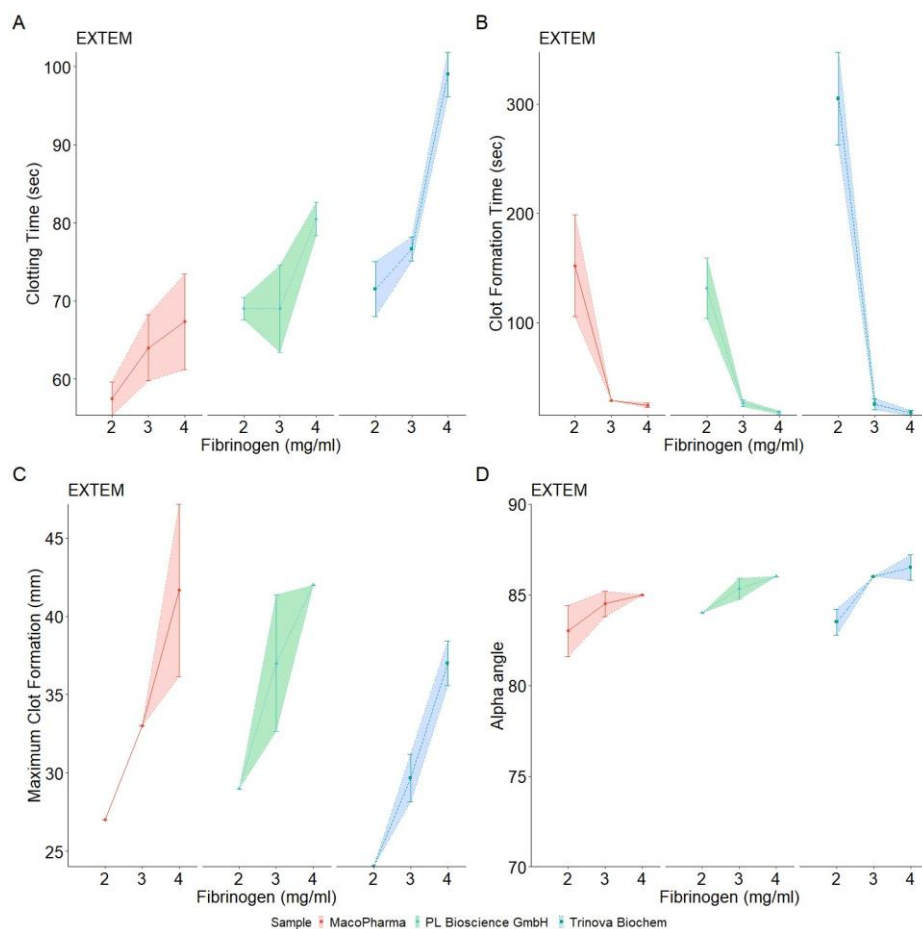


Figure 3.7. EXTEM parameters of commercially available human platelet lysates and effect of fibrinogen supplementation to 2 mg/ml, 3 mg/ml and 4 mg/ml measured by ROTEM.

a) EXTEM Clotting time, b) EXTEM Maximum Clot Formation, c) EXTEM Clot Formation Time, d) EXTEM Alpha Angle (n = 3, measured in duplicate). Mean values with standard deviation shown.

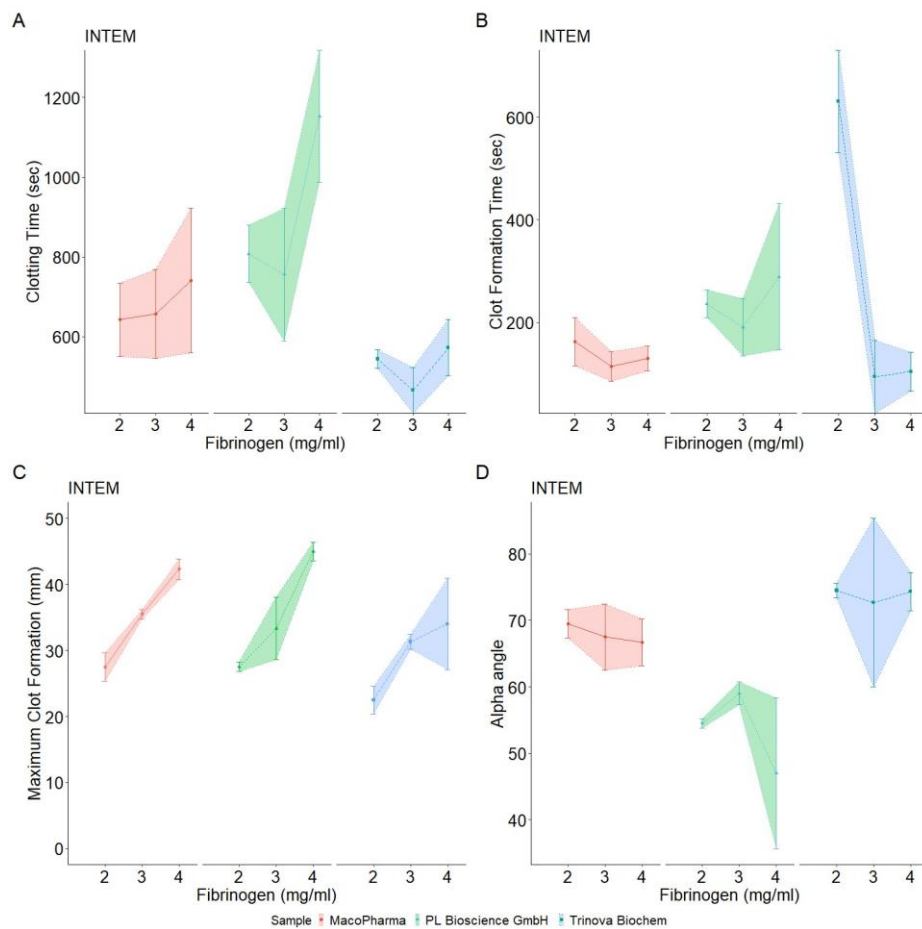


Figure 3.8. INTEM parameters of commercially available human platelet lysates and effect of fibrinogen supplementation to 2 mg/ml, 3mg/ml and 4mg/ml measured by ROTEM.

a) INTEM Clotting time, b) INTEM Maximum Clot Formation, c) INTEM Clot Formation Time, d) INTEM Alpha Angle) (n = 3, measured in duplicate). Mean values with standard deviation shown.

3.3.5 PEP and Commercial Lysates are Enriched in Platelet-Derived Growth Factors

PEP shows significant increases in PDGF-AB (4138.99 pg/ml), VEGF-A (137.94 pg/ml), P-Selectin (57820.77 pg/ml) and NAP-2/CXCL7 (6.4×10^7 pg/ml) compared to PPP (PDGF-AB = 336.31 pg/ml, $p = 0.0022$; VEGF-A = 40.77 pg/ml, $p = 0.0022$; P-Selectin = 28027.42 pg/ml, $p = 0.005$; NAP-2/CXCL7 = 3.1×10^6 pg/ml, $p = 0.0022$). Similar enhancements were observed with HPL (PDGF-AB = 3244.14 pg/ml, $p = 0.024$; VEGF-A = 308.75 pg/ml, $p = 0.024$; P-selectin = 84262.8 pg/ml, $p = 0.0028$; NAP-2/CXCL7 = 102080000 pg/ml, $p = 0.024$) compared to PPP. Interestingly, HPL show similar concentrations of PDGF-AB ($p = 1$) to PEP but have higher concentrations of P-selectin ($p = 0.024$), VEGF-A ($p = 0.024$) and NAP2/CXCL7 ($p = 0.024$). Individual breakdown of platelet-derived factors in HPL is shown in Table 3.2.

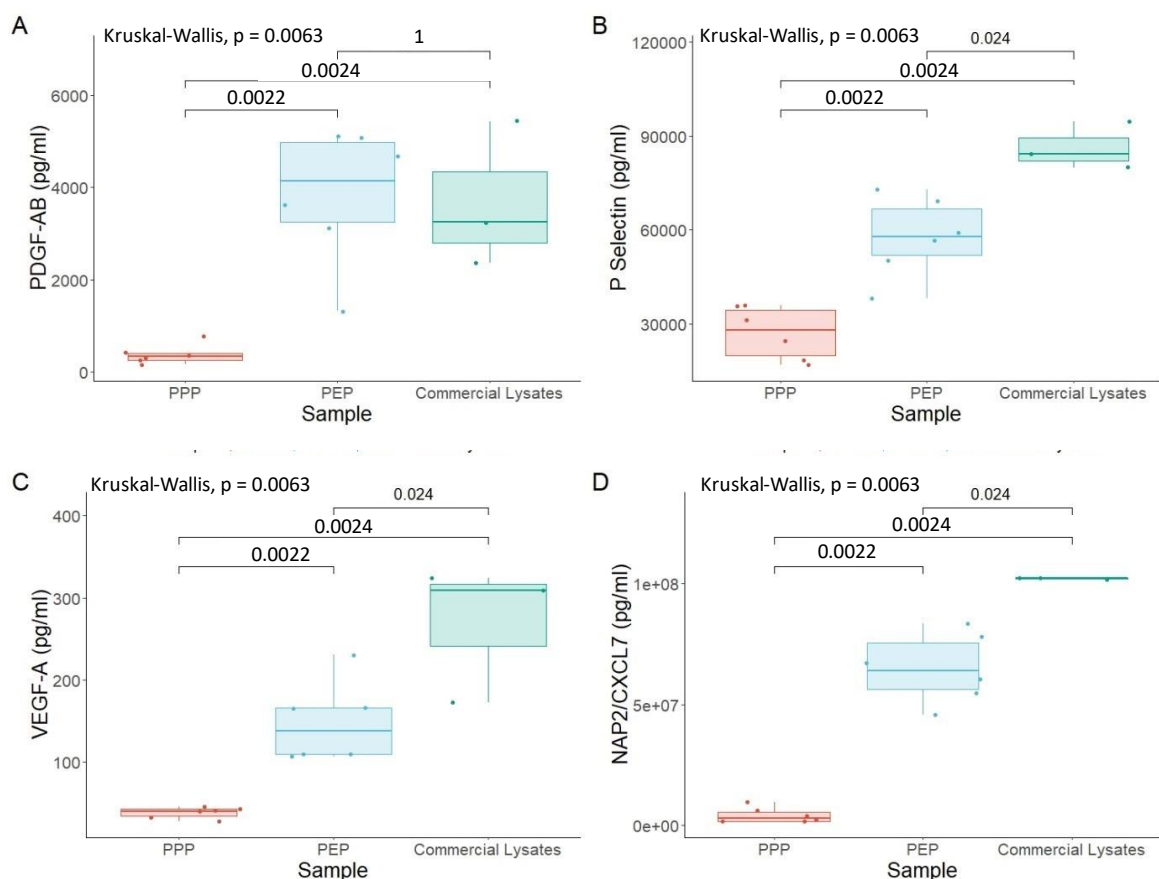


Figure 3.9. Luminex characterisation of PDGF-AB, P-Selectin, VEGF-A and NAP-2/CXCL7 in PPP, PEP and commercially available platelet lysates generated via freeze-thaw cycling.

Kruskall-Wallis tests followed by Wilcoxon sign rank tests for PEP, PRP and PPP; for comparison with commercial lysates Wilcoxon ranks sum tests were used. $n = 6$, measured in duplicate for PEP and PPP. $n = 3$ measured in duplicate for commercial lysates.

Table 3.2. Concentrations of platelet-derived factors (PDGF, P-Selectin, VEGF) in commercial platelet lysates.

Sample	PDGF (pg/ml)	P-Selectin (pg/ml)	VEGF-A (pg/ml)	NAP2/CXCL7 (pg/ml)
MacoPharma	5420.15	8.4×10^4	173.14	1.4×10^8
Trinova Biochem	3244.12	8.0×10^4	308.75	1.0×10^8
PL Bioscience	2359.98	9.4×10^4	323.58	1.0×10^8

3.3.2 PEP shows Enhanced procoagulant activity by Thrombin Generation

Having shown PEP to be acellular, I next determined the procoagulant thrombin generation capacity of PEP using CAT. Figure 3.10 shows a comparison of PRP, PEP and HPLs with PPP and freeze-thawed PPP controls as measured with PRP reagent that detects PS dependent procoagulant activity. Figure 3.10a shows an enhanced leftward shift in thrombin generation dynamics for PEP compared to PPP, PRP, freeze-thawed PPP and HPL. Figure 3.10b shows PEP (255.8 nM) has the highest peak thrombin generation compared to PPP (46.13 nM, $p = 0.0079$), PRP (114.0 nM, $p = 0.0079$) and HPL (72.6 nM, $p = 0.036$). Interestingly, Figure 3.10c shows that PEP (1351.1 nmol/min) ETP does not increase compared to fresh PRP ETP (1268.7 nmol/min, $p = 0.22$); indicating that the total amount of thrombin generated is not altered. PEP ETP (1351.1 nmol/min) was greater than PPP (802.0 nmol/min, $p = 0.0079$) and HPL ETP (1104 nmol/min, $p = 0.036$). As shown in Figure 3.10d, PEP (3.6 mins) lag time is reduced compared to PPP (6.63 mins, $p = 0.0079$) and PRP (6.13 mins, $p = 0.0079$), but was not significantly different to HPL lag time (4 mins, $p = 0.14$). The time to reach peak thrombin generation, (Figure 3.10e) is reduced in PEP (6.94 mins) compared to PPP (13.32 mins, $p = 0.079$) and PRP (14.67 mins, $p = 0.0079$) but not HPL (10.92 mins, $p = 0.39$). Figure 3.10f shows that the start tail of PEP (26.49 mins) is earlier compared to PPP (52.27 mins, $p = 0.0079$), PRP (37.67 mins, $p = 0.0079$) and HPL (41.75 mins, $p = 0.036$). A full breakdown of HPL thrombin generation parameters is shown in Table 3.3. All HPLs showed thrombin generation capacity with MacoPharma and Trinova Bichem showing the shortest lag times and greatest peaks and ETP.

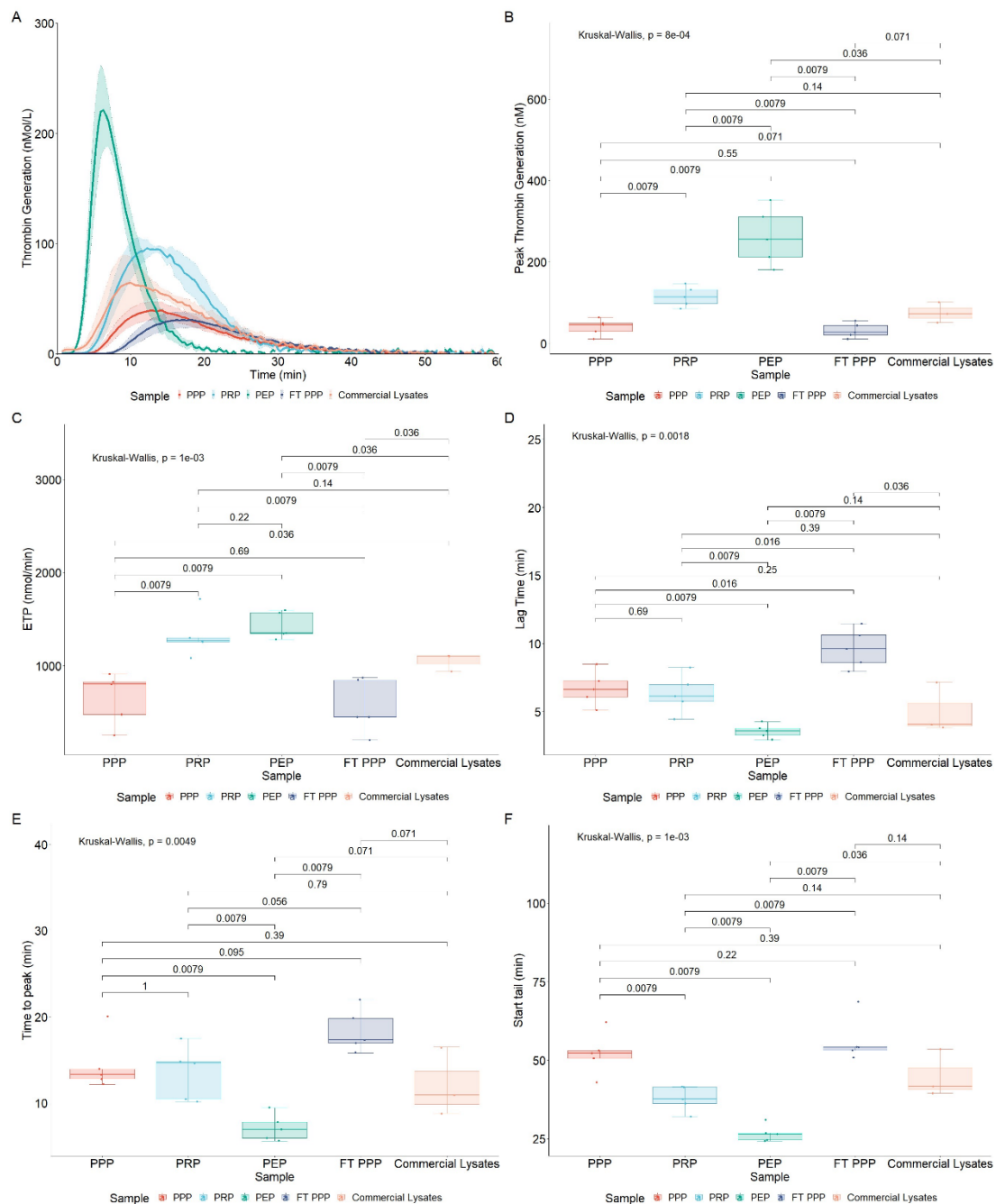


Table 3.3. Thrombin generation parameters for individual commercially available human platelet lysates.

Sample	Lag Time (mins)	ETP (nmol/L)	Peak (nm)	ttPeak (mins)	Start Tail (mins)
MacoPharma	4.09	1104.0	72.6	10.92	41.8
Trinova Biochem	3.83	1106.4	101.3	8.83	39.5
PL Bioscience	7.17	938.5	51.39	16.5	53.5

3.3.3 PEP is Enriched in Procoagulant CD41+ EV

PEP is enriched in functional PS procoagulant activity. This is likely due to PS+ EV, to test this further an additional experiment could have been conducted where PEP was ultracentrifuged to remove EV. I next sought to determine whether ExoView could measure this likely EV population antigenically. Annexin V requires physiological calcium concentration to bind PS. Altering the fluorescent antibody cocktail composition by addition of 2.5 mM Ca^{2+} facilitated visible Annexin V-FITC binding, however fibril like strands were also present across the surface of the chip and the spots. Interestingly fibril formation was especially prominent on CD41 capture as shown in Figure 3.11.

It is likely that the fibril strands were fibrin strands generated by the recalcification of the plasma samples. As GPRP amide inhibits the polymerisation of fibrin this could circumvent this problem. As shown in Figure 3.11 the addition of 15 μM GPRP amide reduced the visual incidence of fibril like stands on the surface of the chip.

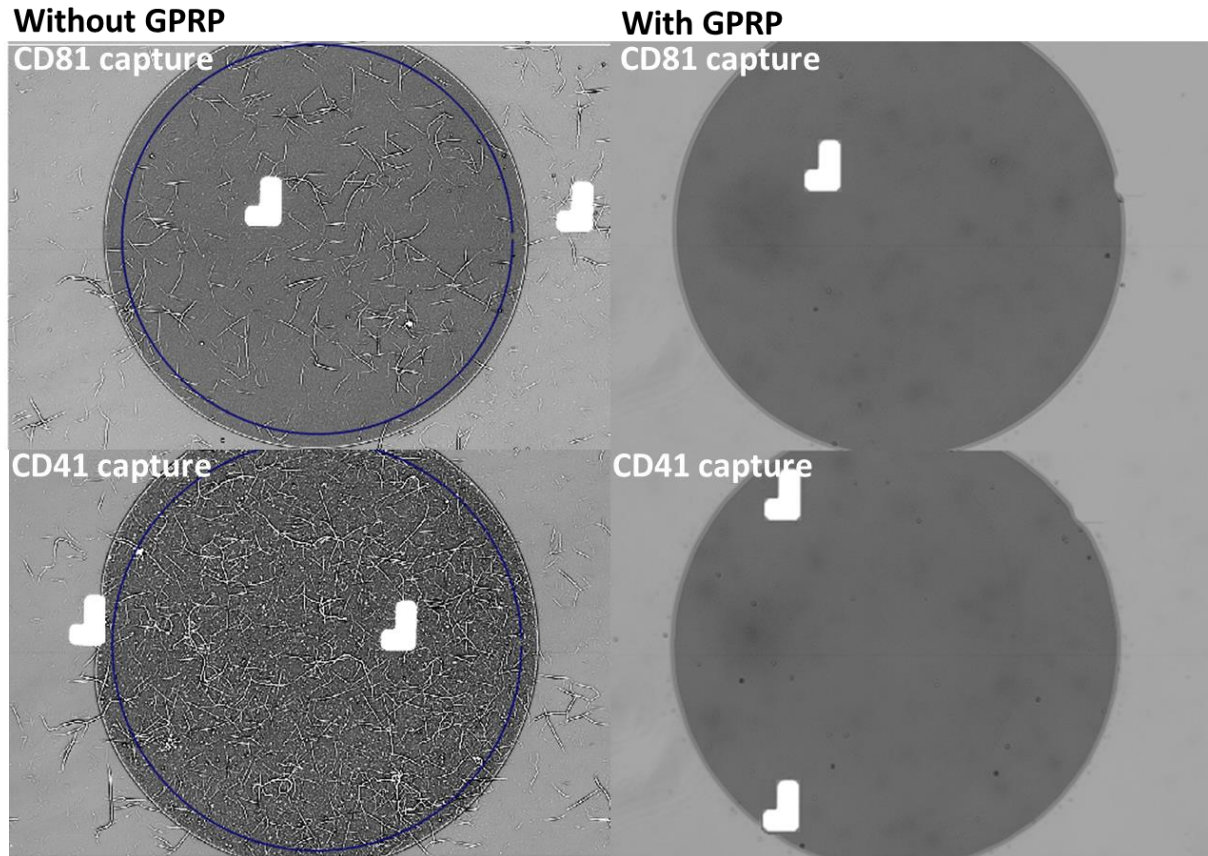


Figure 3.11. Physiological calcium induces fibrin formation in plasma incubated with ExoView tetraspanin plasma chips.

Anti-CD81 and anti-CD41 capture spots show fibrin formation in the presence of plasma samples and calcium (2.5 mM). CD41 shows more fibrin deposition. Both CD41 and CD81 associated fibrin deposition can be avoided through the use of GPRP amide (15 μ M)

Having shown that fibrin formation can be prevented I next tested whether any Annexin V-FITC signal was specific. As Annexin-V only binds PS in the presence of calcium, donor matched PFP was measured with and without calcium in the fluorescent antibody cocktail. As shown below Annexin V-FITC signal is only present in the presence of calcium (Figure 3.14), with significant increases on CD81 (7.3×10^5 vs 6.5×10^4 particles/ml, $p = 0.019$), CD63 (2.1×10^6 vs 4.3×10^5 particles/ml, $p = 0.013$), CD9 (5.9×10^6 vs 6.7×10^5 particles/ml, $p = 0.0087$) and CD41 (2.0×10^7 vs 0 particles/ml, $p = 0.0043$) capture. There was no difference with CD41-PE (Figure 3.12) and CD63-AF647 (Figure 3.13) staining between the annexin protocol and the normal protocol.

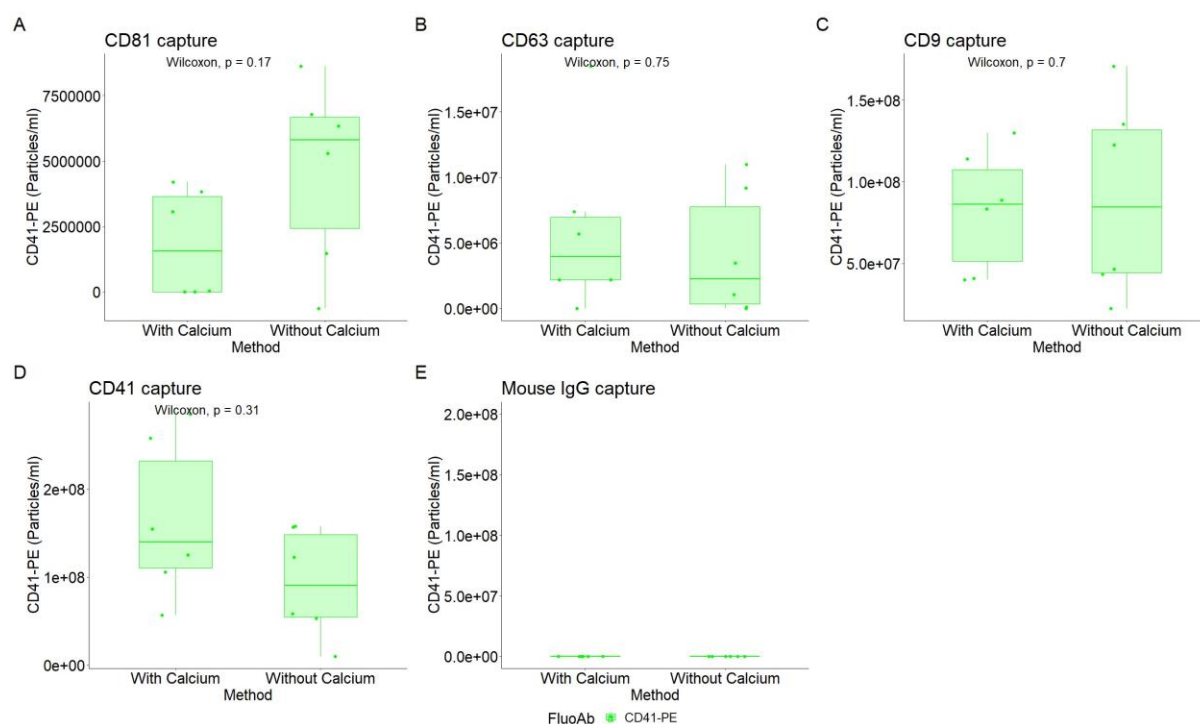


Figure 3.12. Annexin V protocol vs normal protocol for CD41-PE fluorescence.

ExoView fluorescent extracellular vesicle (EV) count (anti-CD41 antibody (CD41-PE)) on platelet-free plasma (PFP) following in-house generated Annexin protocol or non-calcium containing kit provided protocol with results stratified by capture marker: CD81 (a), CD63 (b), CD9 (c), CD41a (d) and Mouse IgG control (e) $n = 6$

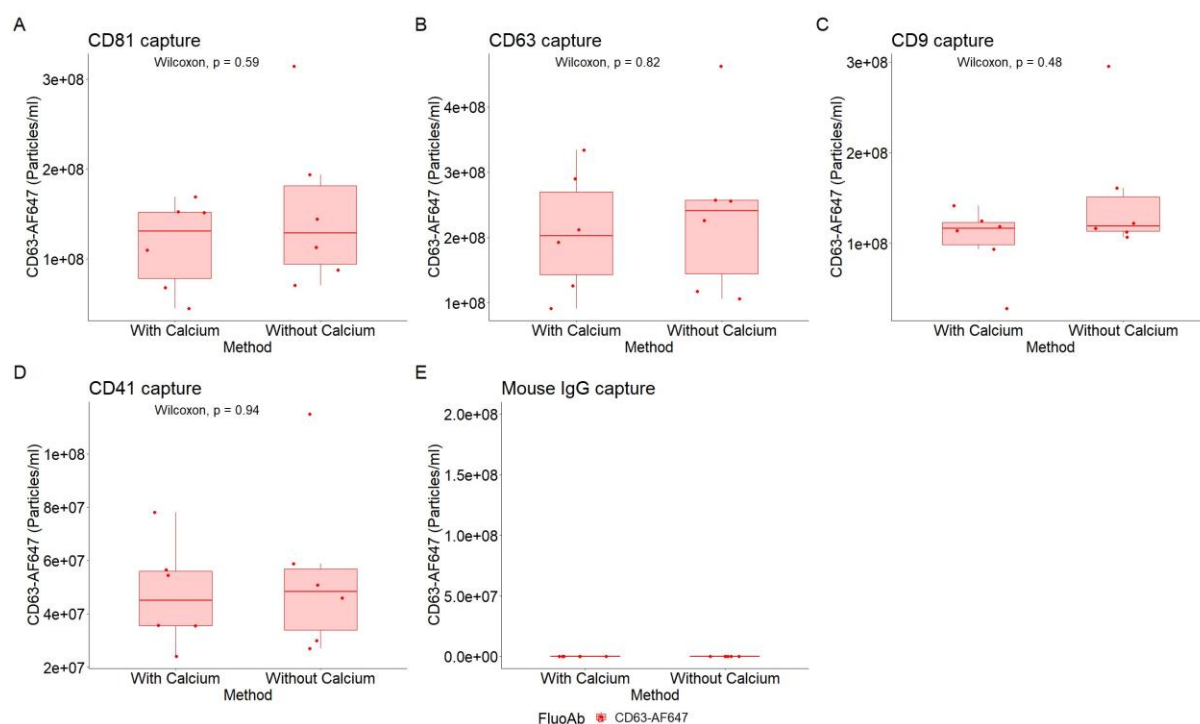


Figure 3.13. Annexin V protocol vs normal protocol for CD63-AF647 fluorescence.

ExoView fluorescent extracellular vesicle (EV) count (anti-CD63 antibody (CD63-AF647)) on platelet-free plasma (PFP) following in-house generated Annexin protocol or normal kit provided protocol with results stratified by capture marker: CD81 (a), CD63 (b), CD9 (c), CD41a (d) and Mouse IgG control (e) $n = 6$

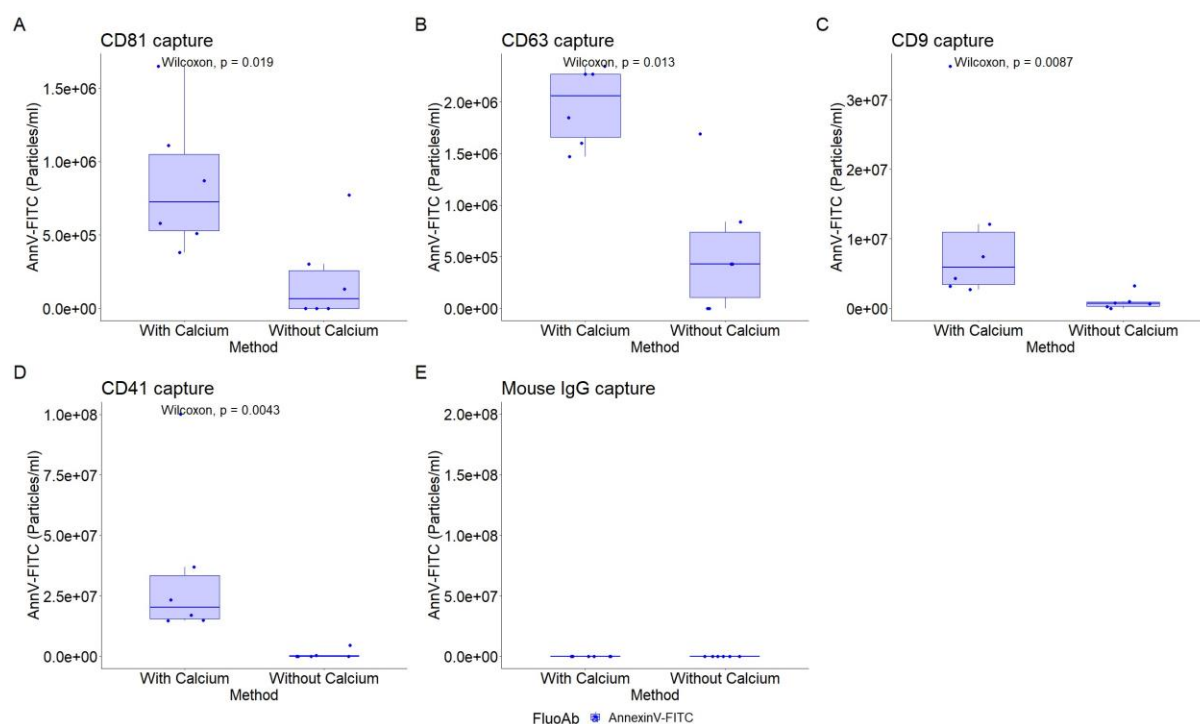


Figure 3.14. Annexin V protocol vs normal protocol for Annexin V-FITC fluorescence.

ExoView fluorescent extracellular vesicle (EV) count (Annexin V-FITC) on platelet-free plasma (PFP) following in-house generated Annexin protocol or normal kit provided protocol with results stratified by capture marker: CD81 (a), CD63 (b), CD9 (c), CD41a (d) and Mouse IgG control (e) $n = 6$

Having established that the Annexin protocol did not alter the EV profile I next sought to use this protocol to investigate the PS expression of EV from PEP compared to PFP. Figure 3.16, 3.17 and 3.18 shows secondary labelling of captured PEP and PFP EV with one fluorescent marker per figure. An alternative way of viewing this data is shown in Figure 3.19. Figure 3.15 shows representative images of PEP vs PFP fluorescent labelling by ExoView.

As expected, there were higher concentrations of PS+ EV captured on CD81 (8.9×10^7 particles/ml), CD63 (6.1×10^8 particles/ml), CD9 (3.1×10^9 particles/ml) and CD41 (6.6×10^9 particles/ml) in PEP compared to PFP (2.35×10^6 particles/ml, 3.25×10^6 particles/ml, 1.3×10^7 particles/ml, 2.4×10^7 particles/ml, $p = 0.0079$) (Figure 3.18). Figure 3.17 shows greater CD63+ EV captured on CD81 (3.0×10^8 particles/ml), CD63 (3.7×10^9 particles/ml), CD9 (4.0×10^9 particles/ml) and CD41 (6.8×10^9 particles/ml) compared to PFP (7.0×10^7 particles/ml, 1.3×10^8 particles/ml, 6.7×10^7 particles/ml, 3.7×10^7 particles/ml, $p = 0.0079$). Figure 3.16 shows greater CD41+ EV capture on CD81 (3.9×10^8 particles/ml), CD63 (1.3×10^9 particles/ml), CD9 (1.2×10^{10} particles/ml) and CD41 (1.1×10^{10} particles/ml) in PEP compared to PFP (4.0×10^6 particles/ml, 3.1×10^6 particles/ml, 4.2×10^7 particles/ml, 1.1×10^8 particles/ml, $p = 0.0079$).

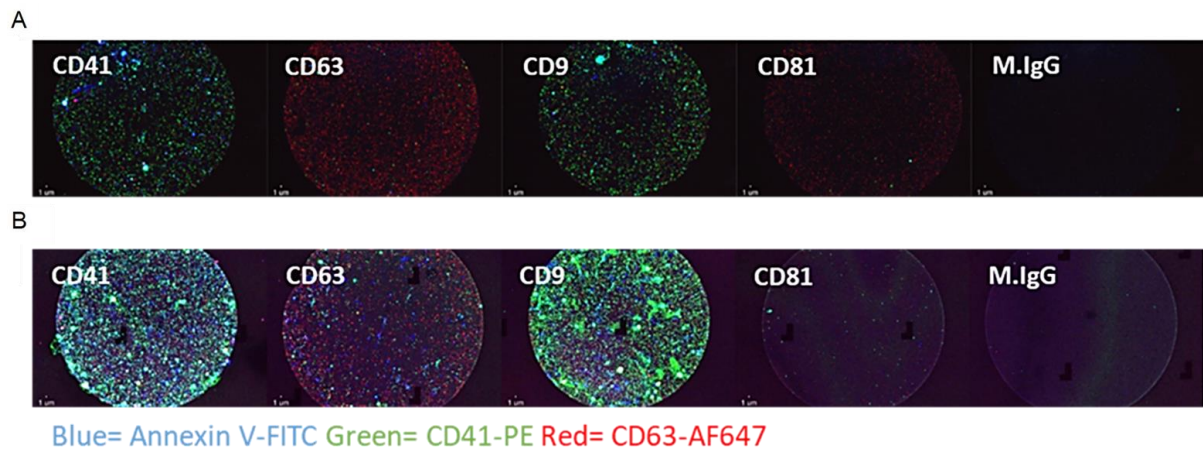


Figure 3.15. ExoView fluorescent images of PEP vs PFP.

a) Representative fluorescent images of PFP measured at a dilution factor of 1:10, a)
 Representative fluorescent images of PEP measured at a dilution factor of 1:250

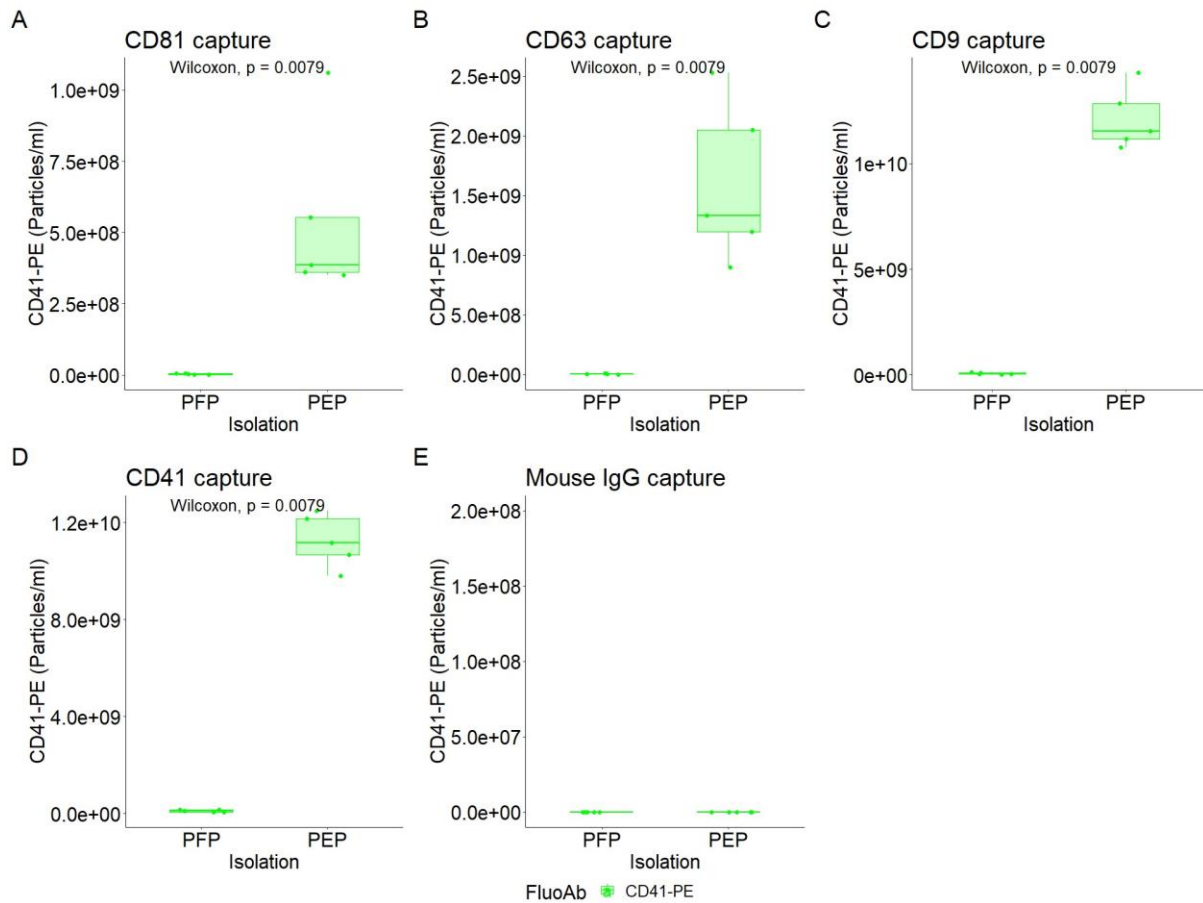


Figure 3.16. EV Characterisation of PFP and PEP CD41-PE.

ExoView fluorescent extracellular vesicle (EV) count (CD41-PE) on platelet-free plasma (PFP) and PEP following in-house generated Annexin protocol with results stratified by capture marker: CD81 (a), CD63 (b), CD9 (c), CD41a (d) and Mouse IgG control (e) n = 5

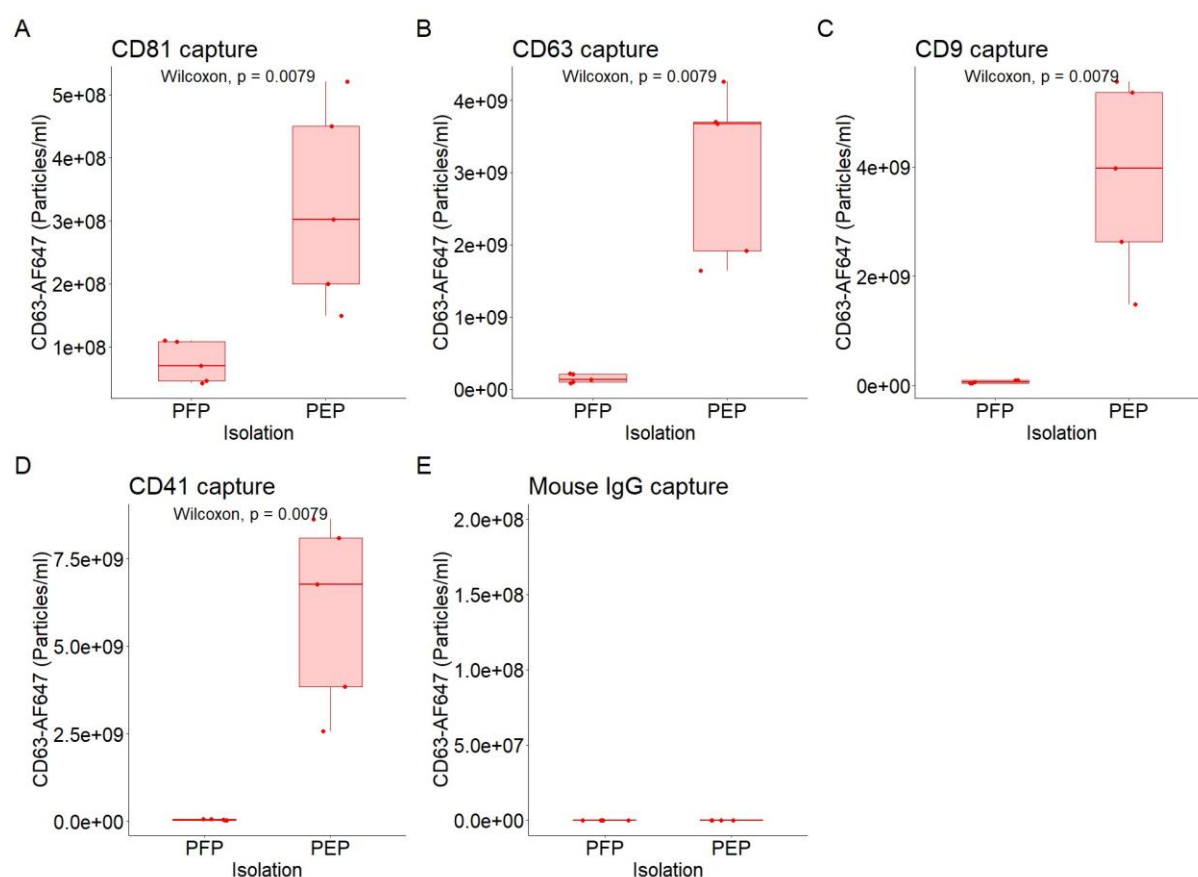


Figure 3.17. EV Characterisation of PFP and PEP CD63-AF647.

ExoView fluorescent extracellular vesicle (EV) count (CD63-AF647) on platelet-free plasma (PFP) and PEP following in-house generated Annexin protocol with results stratified by capture marker: CD81 (a), CD63 (b), CD9 (c), CD41a (d) and Mouse IgG control (e) n = 5

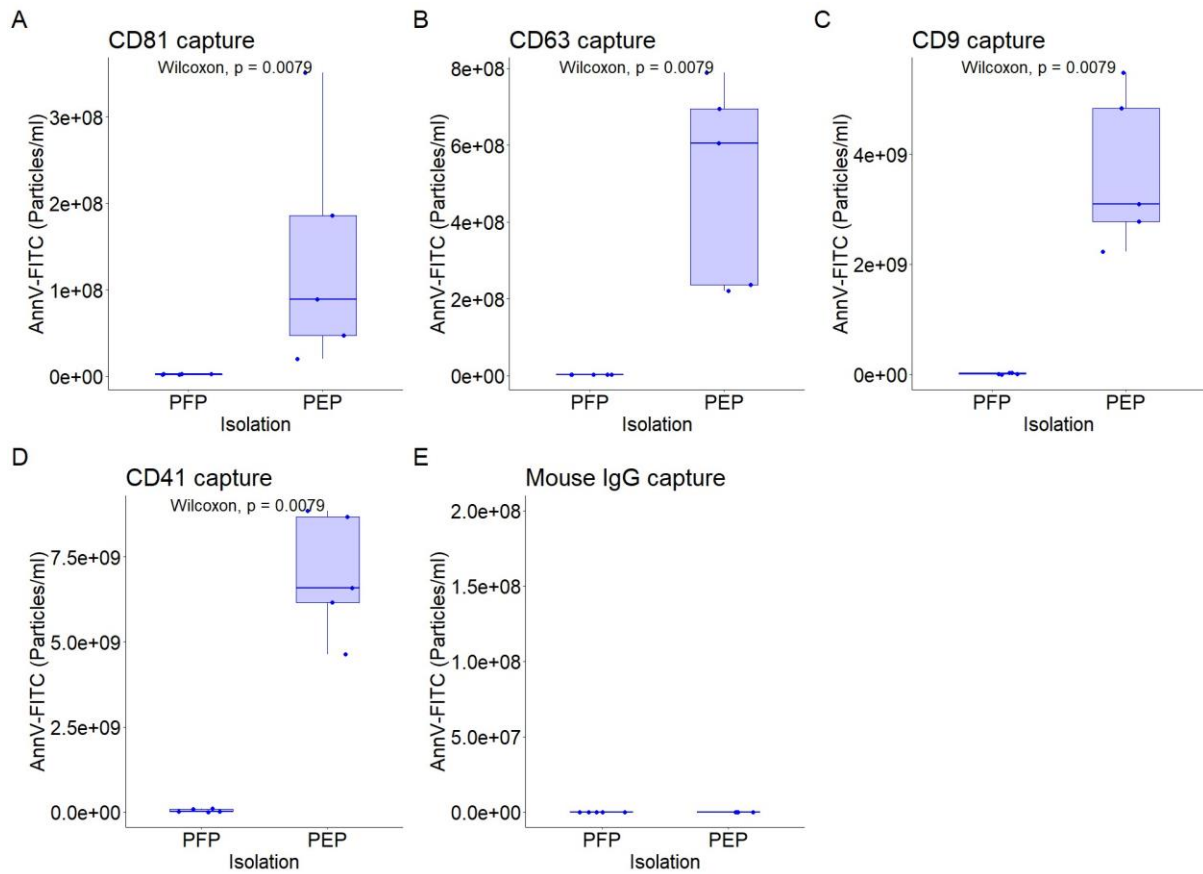


Figure 3.18. EV Characterisation of PFP and PEP Annexin V-FITC.

ExoView fluorescent extracellular vesicle (EV) count (Annexin V-FITC) on platelet-free plasma (PFP) and PEP following in-house generated Annexin protocol with results stratified by capture marker: CD81 (a), CD63 (b), CD9 (c), CD41a (d) and Mouse IgG control (e) n = 5

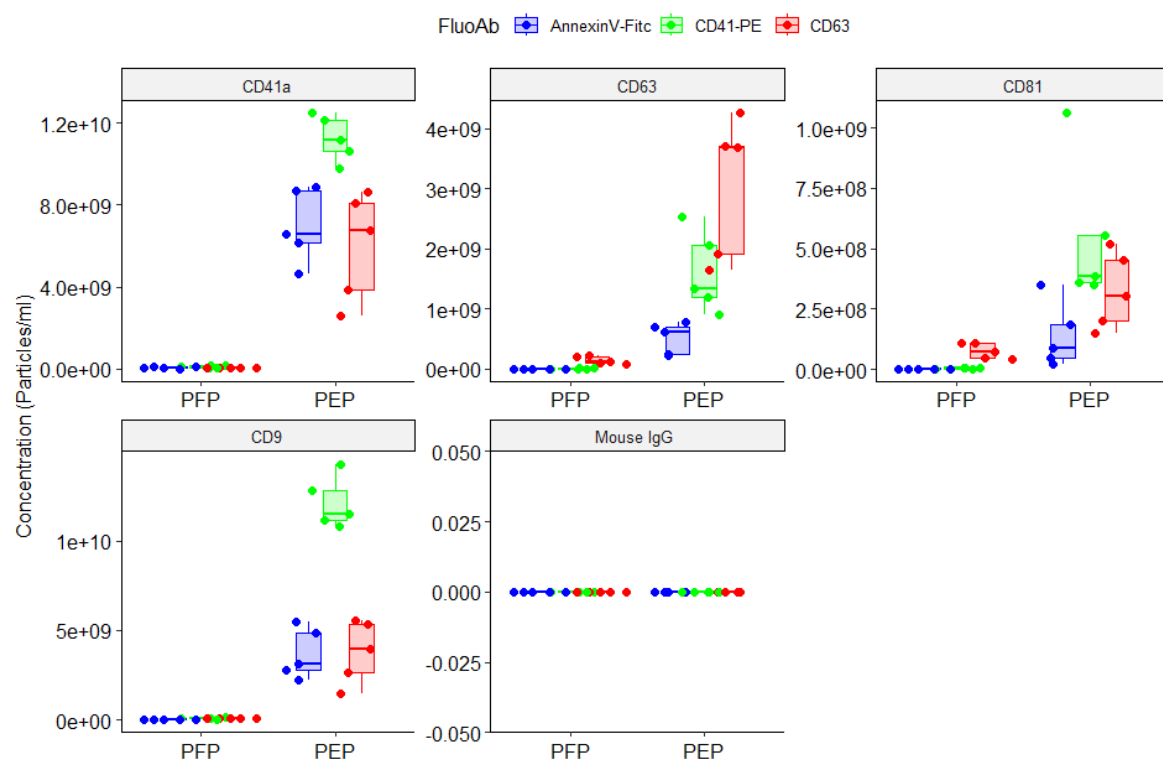


Figure 3.19. Secondary labelling of capture EV from PEP and PFP

ExoView fluorescent extracellular vesicle (EV) count (Annexin V-FITC, CD41-PE, CD63-AF647) on platelet-free plasma (PFP) and PEP following in-house generated Annexin protocol with results stratified by capture marker: CD41a, CD63, CD81, CD9 and Mouse IgG control. n = 5

Table 3.4 shows EV size. The size of PEP EV captured on CD63 (65 nm) and CD9 (80 nm) were not significantly different to PFP EV captured on CD63 (65nm, $p = 0.91$) and CD9 (80 nm, $p = 0.75$). CD41 captured PEP EV (100 nm) were larger than CD41 captured PFP EV (75 nm, $p = 0.012$). CD81 PEP EV (55 nm) were smaller than CD81 PFP EV (70 nm, $p = 0.043$).

Table 3.4. EV size measured by ExoView.

EV sizes are stratified by capture antibody, Wilcoxon sign rank tests were used to determine significance and p values are shown.

Sample	CD41 Capture (nm)	CD63 Capture (nm)	CD9 Capture (nm)	CD81 Capture (nm)
PEP	100	65	80	55
PFP	75	65	80	70
P Value	0.012	0.91	0.75	0.043

3.3.8 PEP generation from Expired Platelet Concentrates

A total of four expired platelet units were received from NHSBT. Two units were 12 days (D12) old upon receipt; the remaining two were 8 days (D8) old upon receipt. Full blood counts on the platelet concentrates upon receipt and throughout processing are shown in Figure 3.20. Also shown, for comparison is PEP and PFP derived from healthy control participants that were run on the same plates. Figure 3.21f shows the thrombin generation of D12 and D8 platelets upon receipt and after undergoing the PEP generation process. A breakdown of thrombin generation parameters is shown in Figure 3.21. Clotting parameters of expired platelets are shown in Table 3.5.

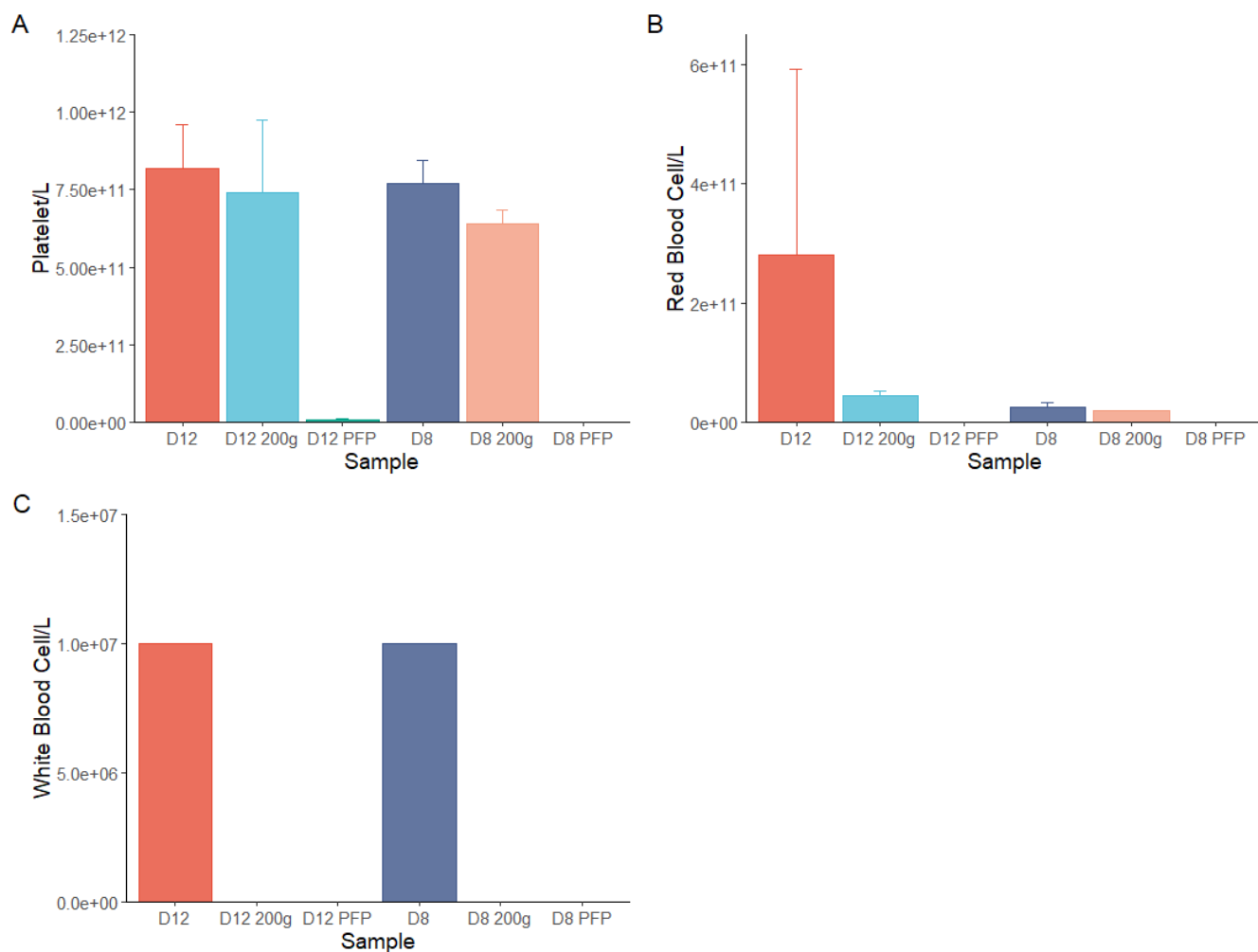


Figure 3.20. Cell Counts from Sysmex XN1000 in expired platelet concentrates.

Day 12 (n = 2) and Day 8 (n = 2) expired platelet concentrates, upon receipt and during processing. a) Platelet counts b) Erythrocyte counts c) Leukocyte counts. Upon receipt platelet concentrates had measurable levels of erythrocyte and leukocyte contamination. Gentle spinning at 200g reduced contaminating cells.

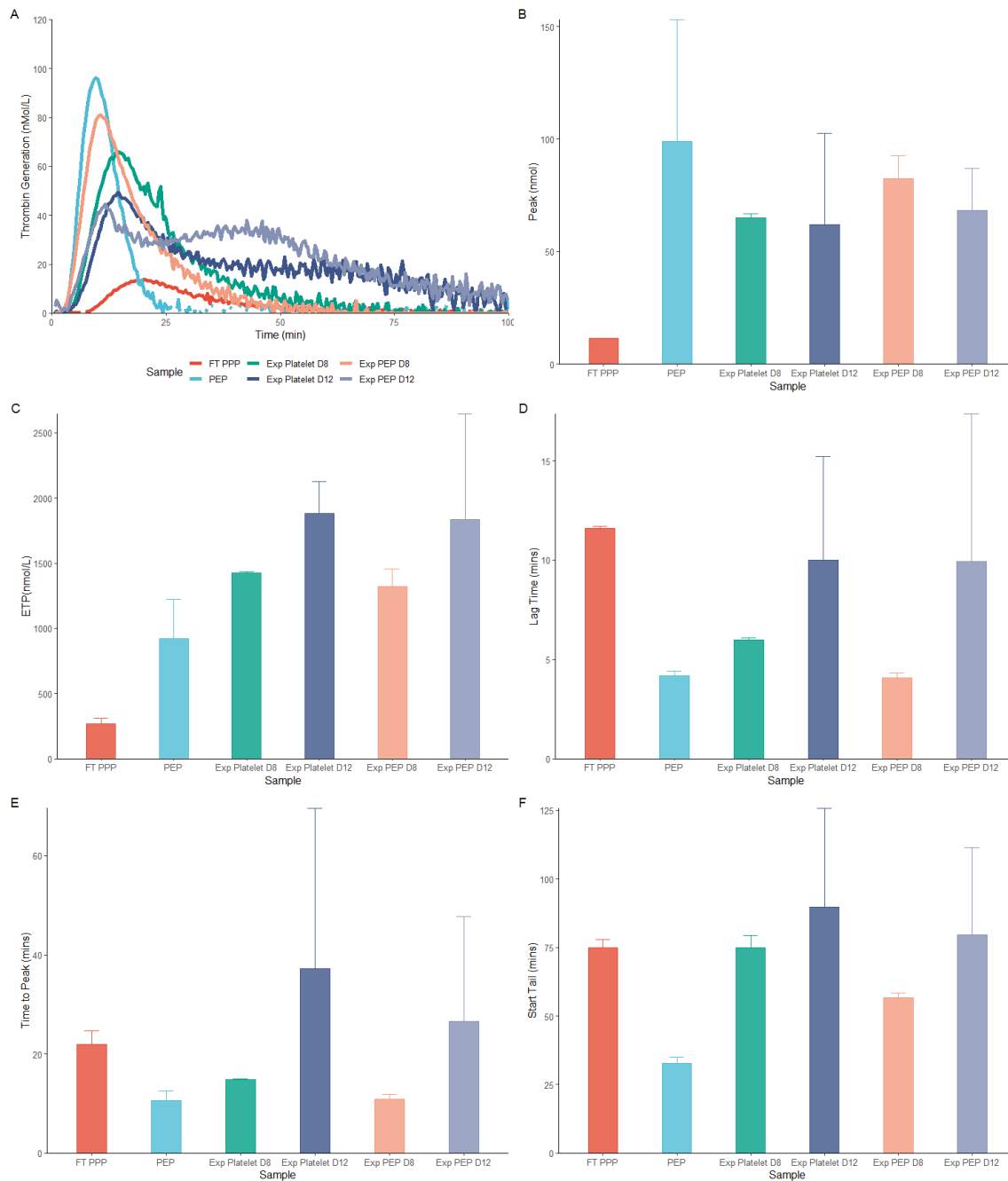


Figure 3.21. CAT Thrombin generation with PRP reagent of expired platelet concentrates.

Day 12 (n = 2) and day 8 (n = 2) expired platelet concentrates, upon receipt and during processing, with inclusion of PFP and PEP derived from healthy volunteers (ran on the same plates (n = 2, measured in duplicate. a) Trace (nmol/L) b) Peak Thrombin Generation (nM), c) ETP (nmol/min), d) Lag Time (min), e) Time to Peak (min), f) Start Tail (min).

Table 3.5. ROTEM Clotting characteristics of expired platelet concentrates.

Day 12 (n = 2) and day 8 (n = 2) expired platelet concentrates, upon receipt and after lysate formation by freeze-thaw cycling (PEP generation).

	EXTEM				INTEM			
Sample	CT (s)	CFT (s)	MCF (mm)	Alpha (°)	CT (s)	CFT (s)	MCF (mm)	Alpha (°)
Expired Platelet Day 12	266.75	997.25	42.25	54	689	465	51.75	55
Expired PEP Day 12	308.25	1317.67	21.5	52.75	851.75	1120	22.75	42.25
Expired Platelet Day 8	72.75	58	25.25	79.5	286.25	32.25	77.25	83.25
Expired PEP Day 8	58.5	864	20.25	74	366.5	0	19.75	60.5

3.3.9 Characterisation of EV from a Scott syndrome Patient

A family presented with a significant history of bleeding including easy bruising, menorrhagia, gastrointestinal bleeding and prolonged bleeding after cuts and poor healing. Family members were recruited to the UK Genotyping and Phenotyping of Platelets (GAPP) study for detailed phenotyping of platelet function, procoagulant activity, EV measurements and genetic studies. Patient III:1 is a 12 year old female with a strong history of a 'probable' hereditary platelet disorder with epistaxis, cutaneous bleeding, bleeding from minor wounds, oral cavity bleeding and menorrhagia. Her mean corpuscular volume (MCV) was slightly reduced (77.2 fL) and has at times been in the normal range. Her haemoglobin (Hb) was normal and other markers of thalassaemia such as mean corpuscular haemoglobin (MCH) have been in the normal range. She was iron deficient with a ferritin of 9.1 g/L (normal range: 13.7 – 78.8). She had an initial platelet count of $301 \times 10^9/L$ and mean platelet volume (MPV) of 10 fL (Table 3.6). Her mother II:2 was 43 years old when recruited to the study with no reported bleeding episodes. She had a platelet count of $284 \times 10^9/L$ and MPV of 9.7 fL (Table 3.6). Individual III:2 (sibling) was also recruited to the study but showed no obvious signs of a bleeding history and was subsequently used for genetic and segregation studies as a control. All other haematological parameters for the family presented were within normal ranges. Family pedigree is shown in Figure 3.22a.

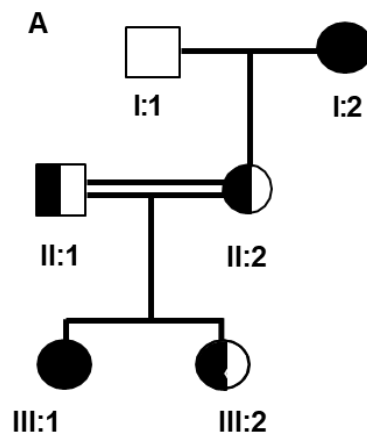


Figure 3.22- Family Pedigree.

A) Pedigree of family showing affected individuals (shaded).

Table 3.6. Scott family members demographics and haematological parameters.

Haematological parameters of patient III:1 and individual II:2. M: male, F: female, WBC: white blood cell, RBC: red blood cell, HGB: Haemoglobin, MCV: mean cell volume, IPF: immature platelet fraction, MPV: mean platelet volume measured with a Sysmex blood cell analyser. *Normal haematological parameters ranges taken from [334].

Parameters	II:2	III: 1	Normal range*
Age	43	12	—
Gender	F	F	—
WBC	6.74	7.52	M 3.91 – 10.90 x10 ⁹ /L F 4.49 – 12.68
RBC	4.36	4.34	M 4.44 – 5.61 x10 ¹² /L F 3.92 – 5.08
HGB	108	107	M 135 – 169 g/L F 119 – 146
MCV	75.9	77.2	M 81.8 – 95.5 fL F 82.9 – 98.0
Neutrophils	3.31	3.05	M 1.8 – 6.98 x10 ⁹ /L F 2.1 – 8.89
Lymphocytes	2.42	3.70	M/F 1.26 – 3.35 x10 ⁹ /L
Monocytes	0.59	0.54	M 0.29 – 0.95 x10 ⁹ /L F 0.25 – 0.84
Platelets	284	301	M 166 – 308 x10 ⁹ /L F 173 – 390
IPF	5.0	5.6	M 0.8 – 6.3 % F 0.8 – 6.2
MPV	9.7	10.0	M 9.3 – 12.1 fL F 9.1 – 11.9
ANO6 variant	c.1943delG	c.1943delG	
	(heterozygous)	(homozygous)	

3.3.9.1 Identification of a novel germline variant in ANO6 in a family with a suspected inherited platelet disorder

The family members were investigated for a possible genetic cause using the R90 bleeding and platelet disorders targeted gene panel performed at Oxford Regional Genetics Laboratories. This detected a novel homozygous frameshift variant in III:1 in the ANO6 gene (c.1943del; p.Arg648Hisfs*23) and was confirmed using Sanger sequencing (Figure 3.22a). Family members II:2 (mother) and III:2 (unaffected sibling) were both heterozygous for the ANO6 variant. This is the fifth ANO6 variant to be reported to date and the most C-terminal positioned 5' to the sixth transmembrane domain of ANO6 (TMEM16F) (Figure 3.23c). The effect of p.Arg648Hisfs*23 on the predicted structure of ANO6 was modelled using AlphaFold26 based on the predicted protein sequence (LUMC Mutalyzer, Normalizer tool) (Figure 3.23b). This revealed a predicted truncation 23 amino acids downstream of the mutation site. The regions within the truncation include four transmembrane helices, a large proportion of the extracellular domain and the C-terminal cytosolic helix including 2/3 of the predicted Ca²⁺ binding sites.

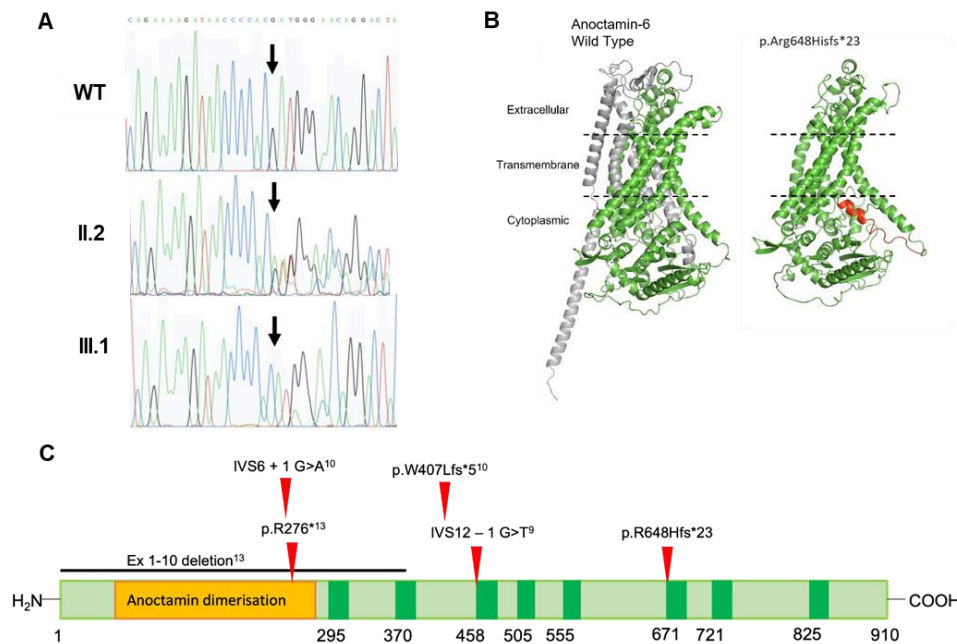


Figure 3.23. Novel mutation in the gene encoding Anoctamin-6 (ANO6/TMEM16F) with a predicted truncation in a Scott syndrome patient.

A) Novel mutation in the gene encoding Anoctamin-6 (ANO6/TMEM16F) following Sanger sequencing (indicated by arrow) with a predicted truncation in ANO6. B) AlphaFold structure prediction of human Anoctamin-6. Dashed line represents the boundaries of the transmembrane region. The residues predicted to be truncated in the p.Arg648Hisfs*23 mutation are highlighted in grey in healthy control image (left) with the altered residues present in the patient highlighted in red (right). C) Schematic of the ANO6 protein showing position of the novel p.Arg648Hisfs*23 amino acid change and predicted truncation (after single base deletion at nucleotide position 1943) along with previously reported mutations. Dark green represents transmembrane domains.

3.3.9.2 Absence of Phosphatidylserine (PS) expression on platelets in Scott syndrome

Flow cytometry analysis of PS+ (% Annexin V positive) platelets following stimulation with ionomycin (10 μ M) or CRP (10 μ g/mL) confirmed the absence of PS expression in patient III:1 (Figure 3.24) compared to II:2 and control.

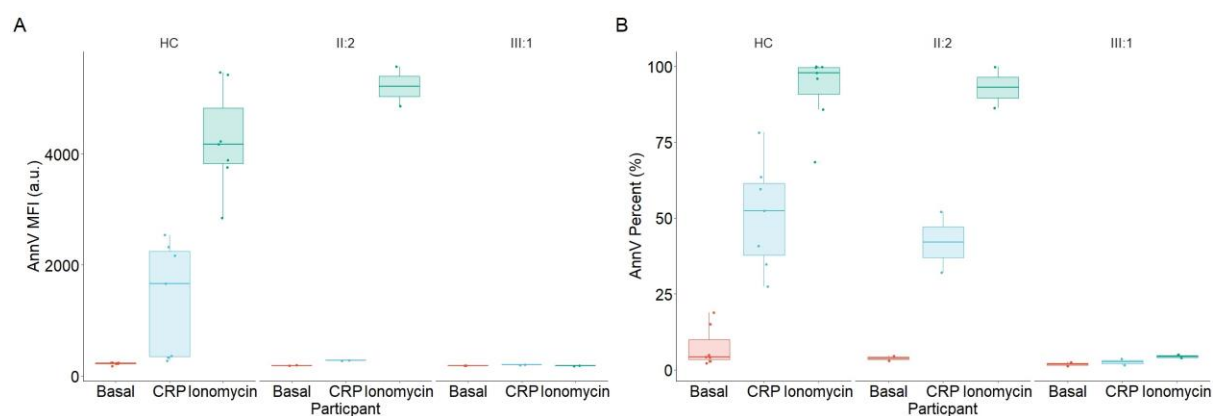


Figure 3.24. PS expression on platelets by flow cytometry.

Healthy control (n = 7), II:2 (n = 2, repeat visit) and III:1 (n = 2, repeat visit) PRP was labelled with annexin-V-FITC following CRP or ionomycin stimulation a) Annexin-V-FITC median fluorescence intensity. b) Annexin-V % platelet expression

3.3.9.3 Reduced thrombin generation in PRP and PFP in Scott syndrome patient

To assess the effect of loss of PS exposure in patient III:1, thrombin generation was analysed in PRP and PFP using CAT (Figure 3.25). Thrombin generation parameters were also measured in PRP and platelet-free-plasma (PFP) using routine calibrated automated thrombography (CAT) with a similar trend in reductions in ETP, peak thrombin generation and increased lag time to thrombin generation in III:1 compared to II:2 and healthy controls.

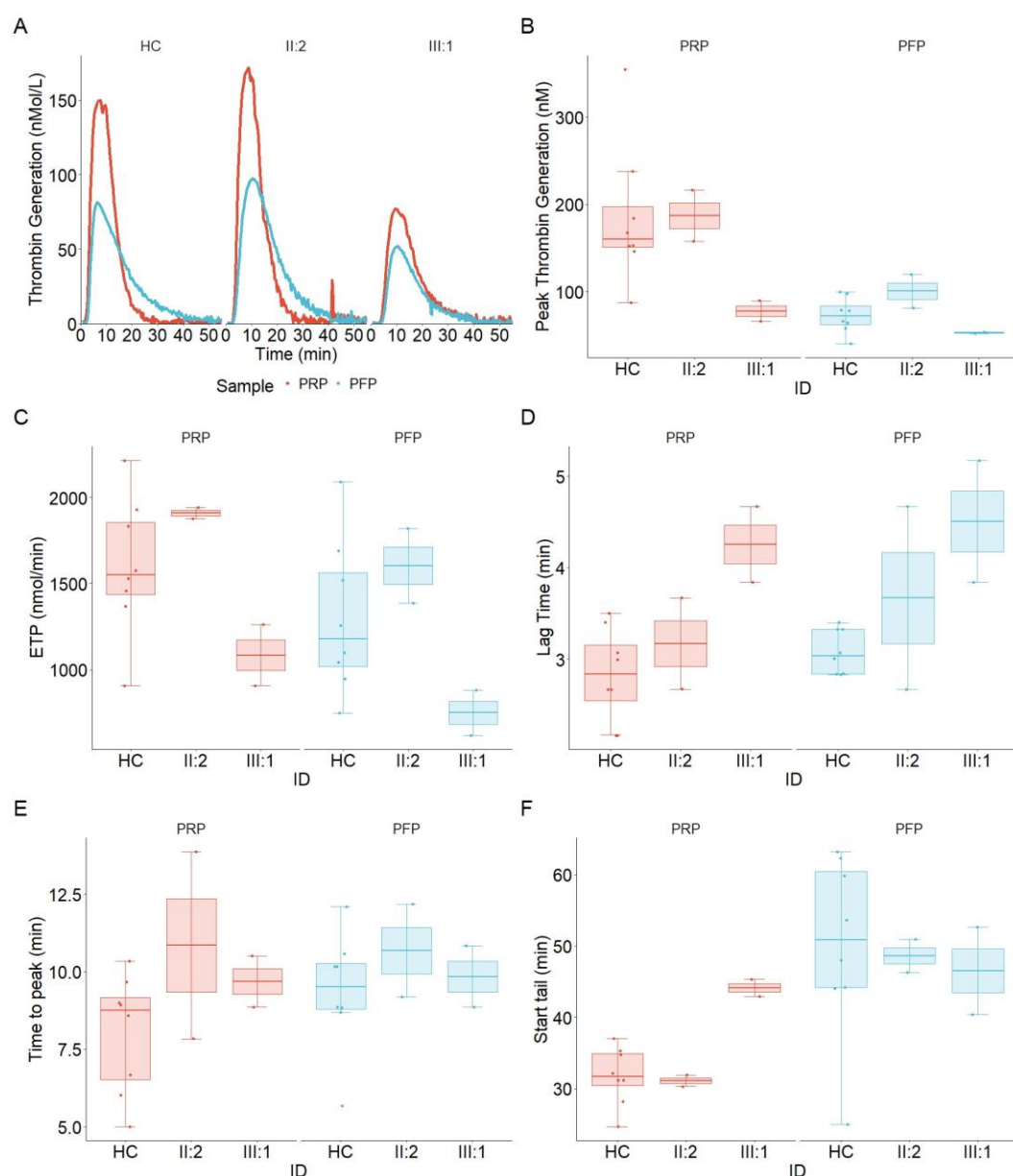


Figure 3.25. Reduced thrombin generation in Scott syndrome.

PRP reagent treatment of PRP and PFP from healthy controls ($n = 8$, measured in duplicate), patient II:2 ($n = 2$, measured in duplicate, repeat visit) and III:1 ($n = 2$, measured in duplicate, repeat visit). a) Trace (nmol/L), b) Peak Thrombin Generation (nM), c) ETP (nmol/min), d) Lag Time (min), e) Time to Peak (min), f) Start Tail (min).

3.3.9.4 Reduced CD41 PS-positive EVs in the plasma and platelet releasate in Scott syndrome

Nanoparticle tracking Analysis (NTA) was used to measure the size and total number of EVs found in plasma samples. The overall size distribution and number of total circulating plasma EVs in III:1, II:2 and control were in all the same orders of magnitude (Figure 3.26 a-b). Plasma EV were analysed by capture on tetraspanin plasma chips arrayed with CD41 capture with secondary labelling with Annexin V-FITC. There were reduced concentrations of CD41 captured PS-positive EVs in PFP plasma of III:1 compared to controls (n = 7) (Figure 3.26c). Furthermore, there were reduced levels of Annexin V positive CD41-captured EVs in the releasate collected from the Scott patient's platelet supernatants after calcium ionomycin (10 μ M) stimulation.

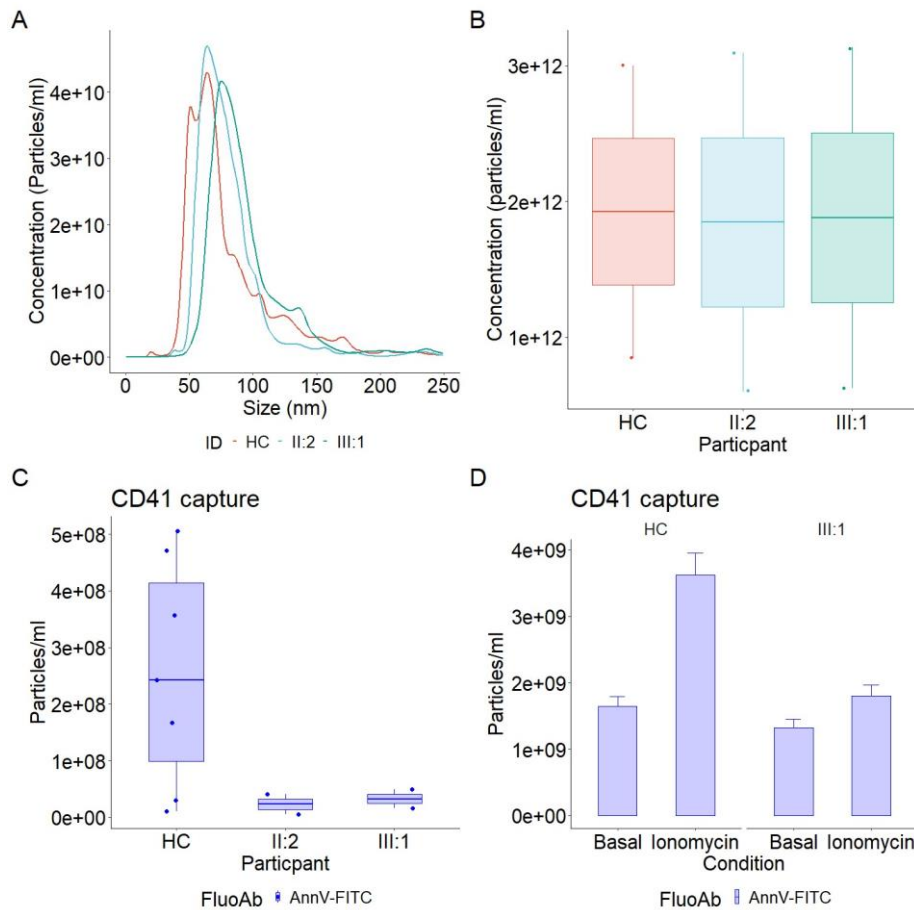


Figure 3.26. Extracellular vesicle characterisation by nanoparticle tracking analysis and ExoView.

a) NTA size and concentration distribution of particles in platelet free plasma (PFP) from healthy controls ($n = 2$ controls, each ran with 5 replicates), II:2 ($n = 2$, repeat sample, each with 5 replicates) and III:1 ($n = 2$, repeat sample, each with 5 replicates). b) NTA particle concentration in PFP from healthy controls ($n = 2$ controls, each ran with 5 replicates), II:2 ($n = 2$, repeat visit, each with 5 replicates) and III:1 ($n = 2$, repeat visit, each with 5 replicates). c) ExoView fluorescent extracellular vesicle (EV) count (Annexin-V-FITC) in PFP from healthy controls ($n = 7$ controls), II:2 ($n = 2$, repeat sample) and III:1 ($n = 2$, repeat sample) following CD41 capture. d) ExoView fluorescent extracellular vesicle (EV) count (Annexin-V-FITC) on CD41 capture in basal unstimulated platelet supernatants and following stimulation with ionomycin ($10 \mu\text{M}$) in healthy control and III:1 ($n = 1$).

3.4 Discussion

PEP is a platelet enhanced plasma that contains high levels of procoagulant PEV with significantly enhanced procoagulant activity and thrombin generation dynamics compared to donor matched PRP and PPP (Figure 3.10). PEP can be generated from platelets from fresh whole blood (PRP), with preliminary evidence indicating an optimised procedure that could also be utilised on platelet concentrates. PEP also contains high amounts of platelet derived growth factors (Figure 3.9) which could aid in wound healing and immune cell recruitment [335]. PEP also successfully restored clotting times in an *in vitro* model of dilutional coagulopathy and therefore forms a novel candidate resuscitation fluid (Figure 3.5 and 3.6). The MCF of PEP however, is not as high as PRP and is more analogous to PPP. Platelets are incorporated into the clot and in their absence clot strength declines. PEP treatment of the *in vitro* dilutional coagulopathy model did not restore MCF but did successfully restore clotting time. The clotting and thrombin generation data together suggest PEP would be useful in accelerating the formation of a stable clot. PEV have been shown to have positive effects on platelet function as assessed by aggregometry [336]. PEV have also been shown to exhibit non-haemostatic effects on the vessel wall, inflammation and vascular tone which may be beneficial for the treatment of haemorrhage and trauma [162].

The potency of PEV has recently been demonstrated in mouse models of severe haemorrhage, where PEV alone restored mean arterial blood pressure, plasma protein concentration, lactate levels and the base deficit compared to vehicle or fresh platelet administration [147]. A recent publication suggests a PEV rich platelet lysate

generated via sonication and suspension in a balanced salt solution could be used as a topical treatment to treat bleeding in trauma patients [337]. Saline has repeatedly been shown to be less efficacious than plasma for fluid replacement in trauma patients, where it can promote dilutional coagulopathy and contribute to metabolic acidosis[338-340]. In contrast to other PEV rich solutions [147, 337, 341-343] PEP is suspended in plasma and contains high levels of PAI-1 which may also counteract the frequent hyperfibrinolysis often observed in the coagulopathy of trauma.

EV are historically challenging to measure by direct measurement in complex biofluids such as plasma. I utilised a new surface-molecule affinity EV capture with secondary labelling of captured EV using the ExoView platform and minimal sample manipulation. This vesicle phenotyping confirms that PEP EV are largely platelet derived and procoagulant (CD41+ and PS+). The ExoView results also confirmed the functional output from the CAT experiments, namely that there are more PS-expressing EV in PEP than in PFP.

Using the PEP procedure on NHSBT platelet units could also provide an avenue for extending the potential clinical utility of platelets and aid in reducing wastage [344]. As HPL manufacturing already exists, it may be possible to generate an off-the-shelf resuscitation fluid using an existing workflow. However, despite the strong capacity for thrombin generation in HPL, clotting was impaired in commercially available lysates. However, I have shown fibrinogen supplementation also restores clotting. Other processes are likely involved in commercial HPL generation that impede clotting as the same impairment is not observed in 8-day old NHSBT platelet units. Certainly, HPL have been optimised for cell culture and this is reflected in the significantly greater

concentrations of P-Selectin, VEGF-alpha and NAP2/CXCL7 present in HPL compared to PRP-derived PEP. It is feasible that the PEP generation procedure may be more beneficial if started earlier. A time course following platelet ageing and subsequent PEP composition and functional activity will be necessary to determine the optimal timepoint for PEP generation. A future PEP-like product would undergo full proteomic analysis, measurement of serotonin, plasminogen activator 1 (PAI-1) and polyphosphate[345]. PEV interaction with leukocytes, the endothelium and platelets will also be important parameters [164].

An additional challenge with EV based therapeutics is establishing dosage and ensuring a standardised final product as covered in in this cited position paper [346]. A final PEP preparation derived from platelet units could be investigated through *ex vivo* treatment of blood from trauma patients with coagulopathy, followed by investigation in an *in vivo* small animal model of trauma. An additional method that could be used is to spike PS+ liposomes into plasma, this approach would benefit from the fact that liposomes are easy to make in bulk, are easy to standardise. However, this approach would not extend the lifecycle of platelets for resuscitation purposes.

I also describe a new family with Scott syndrome and a history of excessive bleeding. The family harbours a novel homozygous frameshift variant in the ANO6 gene, a single base deletion at 1943 (c.1943del). This mutation induces a frameshift in the sequence and is predicted to give a premature stop codon and protein truncation 23 amino acids downstream of the mutation (p.Arg648Hisfs*23). The AlphaFold structure prediction of p.Arg648Hisfs*23 Anoctamin-6 revealed a truncation 23 amino acids downstream of the site mutation. This 'lost' region includes the four transmembrane helices, a large

proportion of the extracellular domain and the C-terminal cytosolic helix including 2/3 of the predicted Ca^{2+} binding sites and therefore predicted the mutated protein to be functionally inactive. This is only the fifth ANO6 variant to be reported to date and the most C-terminal, positioned 5' to the sixth transmembrane domain of ANO6 (TMEM16F). It is only the 4th unrelated family to be reported with Scott syndrome to date which may be an underdiagnosed condition. All other cases in the literature are compound heterozygotes [347, 348] apart from 1 other study with a homozygous splicing mutation [149]. Due to the complete loss of PS exposure in our patient, the full-length protein is functionally defective indicating the importance of this C-terminal portion of the protein for mediating PS exposure. Structural studies have identified movement of transmembrane region 6 (TM6) of TMEM16F (ANO6) as being important for causing membrane thinning downstream of Ca^{2+} binding [349, 350]. These conformational changes are important for PS translocation to the outer leaflet and so provides a possible mechanism for the phenotype seen here. ANO6 is an essential component for the Ca^{2+} -dependent exposure of PS on the platelet surface, necessary to provide an interaction site for the assembly of the tenase and prothrombinase complexes[149] Therefore, disrupting this essential gene/protein can lead to a significant bleeding phenotype in human patients as procoagulant thrombin generation, and subsequent downstream processes (e.g. fibrin formation) are reduced. However, thrombin generation is not currently routinely performed in hospital laboratories and so defects in this process may be under-reported [351]. I confirmed the homozygous patient exhibited ablated platelet PS expression using flow cytometry. Using CAT I was also able to demonstrate a marked reduction in PS driven thrombin generation in PRP. It is also thought that in Scott syndrome, PS-exposing EV are not

able to be shed by platelets, with the first observation being a marked reduction in PEV release from a Scott syndrome patient in 1989 [352, 353]. I show that thrombin generation of PFP in the patient is also reduced suggesting a reduction in unchallenged circulating PS-expressing EV. I show with ExoView, using the Annexin protocol established for the PEP research, that our patient's PFP has reduced CD41 captured PS-expressing EV compared to healthy controls. I also show that ionomycin stimulation of patient platelets does not lead to an increase in PS-expressing EV. Interestingly, the heterozygote parent II.2 appeared to have an intermediate phenotype with low MFI PS expression following CRP stimulation of platelets and low basal PS expressing CD41 captured EV as measured by flow cytometry. It would be interesting to return to this participant and assess whether they express both truncated ANO6 and full length ANO6 which may explain this phenotype.

Inherited platelet disorders comprise an extremely heterogeneous group, making genetic diagnosis challenging, especially in those with normal platelet counts [354, 355]. Some patients experience frequent and debilitating bleeding episodes throughout their lifetime. Therefore, a precise genetic diagnosis provides significant clinical benefit for the patients and offers the possibility of accurate counselling and clinical management. Furthermore, routine platelet function testing does not usually measure PS exposure or platelet procoagulant activity, which could ultimately lead to misdiagnosis and may partially explain why Scott syndrome is so rare and potentially underdiagnosed. In conclusion, our study highlights the importance of including ANO6 gene in diagnostic genetic screening for patients with significant bleeding and undiagnosed platelet disorders, particularly for those with a normal platelet count

[355]. I confirm that the procoagulant PS externalisation defect in Scott syndrome platelets extends to their released and circulating EV.

Limitations to the PEP work include the scaled-down approach, the model of dilutional coagulopathy used, which could be improved by simulating metabolic acidosis and strengthened with an additional control of diluted blood measured at 37°C to assess the relative contribution of hypothermia to the model.

Scott syndrome research is limited by the low number of patients available to study, I was fortunate to receive two separate visits from the family to receive blood samples and perform many experiments on both sets of samples. For future patients it would be interesting to confirm the absence of PS on their PEV by a broader range of agonists including following GPVI stimulation. Although Scott syndrome is rare there are alternative approaches to studying it, including the use of inhibitors (niclosamide [356]) of phospholipid scramblase TMEM16F and the availability of mouse models that recapitulates Scott syndrome phenotype [150]. Validation of Scott syndrome models would be valuable to study the mechanisms of PS exposure on activated platelets and may contribute to the development of new anti-platelet therapies.

Chapter 4. Measurement of tissue factor expressing extracellular vesicles

Some of this work has been published under the title: “Detection of tissue factor positive extracellular vesicles using the ExoView R100 System” Price *et al*, 2023

DOI: [10.1016/j.rpth.2023.100177](https://doi.org/10.1016/j.rpth.2023.100177)

My contribution to this publication included writing the manuscript and performing the vast majority of the experiments, Prof Nigel Mackman and Dr Yohei Hisada provided tissue factor activity data for ovarian cancer patients, but all other data and experiments were designed and carried out by me.

4.1 Rationale

TF EV detection is difficult with contradictory reports in the literature. Using established antibodies and testing any potential measurement system on established controls is recommended prior to testing relevant clinical cohort samples.

The TF-VIIa extrinsic pathway is considered the most important physiological activator of the clotting cascade. The expression and regulation of procoagulant TF is crucial for controlling thrombin generation and clotting. Historically, TF was exclusively thought to be expressed outside or “extrinsic” to the circulating blood and only exposed to the blood after tissue injury. Although it is well known that circulating monocytes can be induced through activation to express TF, the expression of TF on other cells (e.g. neutrophils, platelets and endothelium) is more controversial. Despite this, the concept of circulating TF has become more popular whereby circulating EV have been described to express TF in health and disease. This pathway is important in thrombosis (e.g. cancer and cardiovascular disease) and VTE and may also be important for the transport of TF into growing thrombi whereby TF can activate clotting to stabilize the thrombus through thrombin and fibrin generation locally. As TF is a trace protein, direct measurement of TF antigen on either cells and/or EV has been historically challenging. Direct antigenic measurement of TF in plasma has been reported, however, the low abundance of TF (<20fM [193], <2 pM [217]) in healthy individuals typically falls below or is near the detection limit of these assays [207, 217-219]. Furthermore, background signal is high when measured in plasma [73, 217, 220]. These issues are further compounded by the specificity of the antibodies used and the type of TF standard employed [221]. On the contrary, TF activity assays are

highly sensitive [222] but typically require the isolation of EV from plasma [223]. Furthermore, TF activity assays cannot measure encrypted TF which may form a pool of physiologically latent TF activity.

Given the potential of the ExoView technology for measuring and capturing specific EV populations I therefore hypothesized that by using the customizable ExoFlex chips would provide an opportunity to test whether ExoView technology can measure a rare TF EV population in health and disease in plasma samples.

4.2 Aims

- Investigate and optimise the potential of ExoView technology for measurement of TF-expressing EV using appropriate capture and detection anti-TF antibodies within customized ExoFlex chips and ExoView technology.
- Validate using controls that include relevant negative and positive plasma controls generated with and without *in vitro* LPS stimulation of normal whole blood (a well validated protocol for TF EV formation) [203] and in supernatants from a pancreatic cancer cell line BxPC-3 as an additional positive control and appropriate plasma samples from patients with traumatic injury and ovarian cancer.
- Compare, contrast and validate results generated by ExoView to a well-established TF activity assay [222].

4.3 Results:

4.3.1 TF expression by LPS stimulated monocytes

In order to determine whether ExoView can detect TF EV I generated a set of positive controls by stimulating monocytes within whole blood using LPS, this allowed for

optimization and testing of TF antibodies prior to use with ExoView and confirmation of activation by flow cytometry.

Figure 4.1a shows the gating strategy used to identify TF+ monocytes. Following gating based on forward and side scatter, doublets were removed and CD14-FITC+ positive events were selected. 2500 CD14+ monocytes were acquired. Whole blood was stained with monoclonal TF(IIID8)-AF647. As TF(IIID8) was conjugated to AF647 in-house I used LPS stimulation of whole blood to generate TF-expressing EV to confirm that the antibody provided a signal. LPS stimulation is a well-established method to generate TF+ monocytes [220]. Figure 4.1b shows that LPS+ whole blood gave an increase in the percentage of TF+ monocytes, measured with IIID8-AF647, from 21.3% to 76.7% ($n = 8$, $p < 0.001$). Figure 4.1a shows the median fluorescence intensity with IIID8(TF)-AF647 increases following LPS stimulation from 1449 a.u. to 8329 a.u. ($p = 0.0019$). These data indicate that TF(IIID8)-AF647 conjugation was successful and could optimally detect TF expression on LPS stimulated monocytes. I also tested VIC12-AF647 (Figure 4.1c and d) and 5G9-AF647 (Figure 4.1e and f) detection of TF expressing monocytes but determined that IIID8 gave the strongest signal. PFP samples were also prepared and aliquots frozen for measurement of TF EV below.

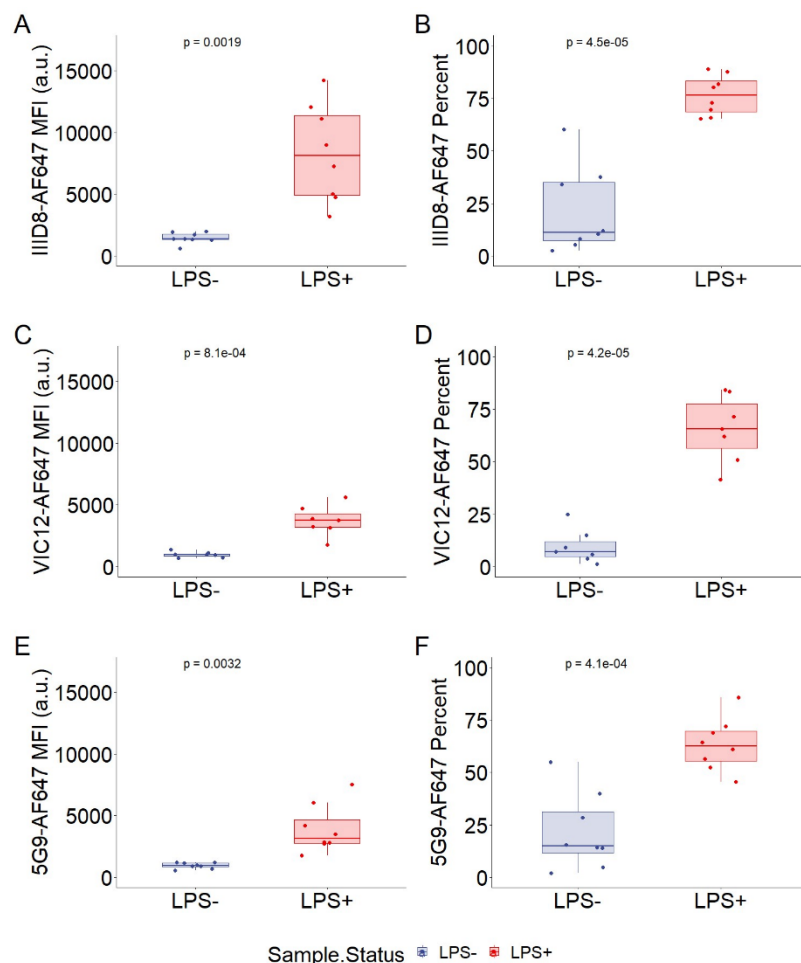


Figure 4.1. TF expression by LPS stimulated monocytes.

a) Anti-TF antibody (IIID8-AF647) median fluorescence intensity, $p = 0.0019$, $n = 8$. b) Anti-TF antibody (IIID8-AF647) % expression on monocytes, $p = 0.000045$, $n = 8$. c) Anti-TF antibody (VIC12-AF647) median fluorescence intensity, $p = 0.00081$, $n = 7$. d) Anti-TF antibody (VIC12-AF647) % expression on monocytes, $p = 0.000042$, $n = 7$. e) Anti-TF antibody (5G9-AF647) median fluorescence intensity, $p = 0.0032$, $n = 8$. f) Anti-TF antibody (5G9-AF647) % expression on monocytes, $p = 0.000042$, $n = 8$.

4.3.2 Characterisation of BxPC-3-derived EVs

To confirm that BxPC-3 cells were secreting EV I used NTA and ExoView to measure culture supernatants. NTA was used for the purpose of checking that EV were present via a cheaper method prior to utilising ExoView chips which are more prohibitive in terms of cost.

Figure 4.2 shows NTA characterisation of EV derived from the culture supernatant of serum-starved BxPC-3 cells compared to serum-free media control. Figure 4.2a shows the size and concentration distribution of EV. Figure 4.2b shows BxPC-3-derived EV from culture supernatant (7.1×10^9 particles/ml) compared to serum-free media control (3×10^5 particles/ml) ($p = 0.0022$). Figure 4.2c shows that BxPC-3 derived EV isolated from culture supernatant are larger than those of the serum-free media control ($p = 0.005$). Although, NTA is not capable of directly identifying TF+ EV, this confirms that there are EV present in BxPC-3 cell culture supernatant.

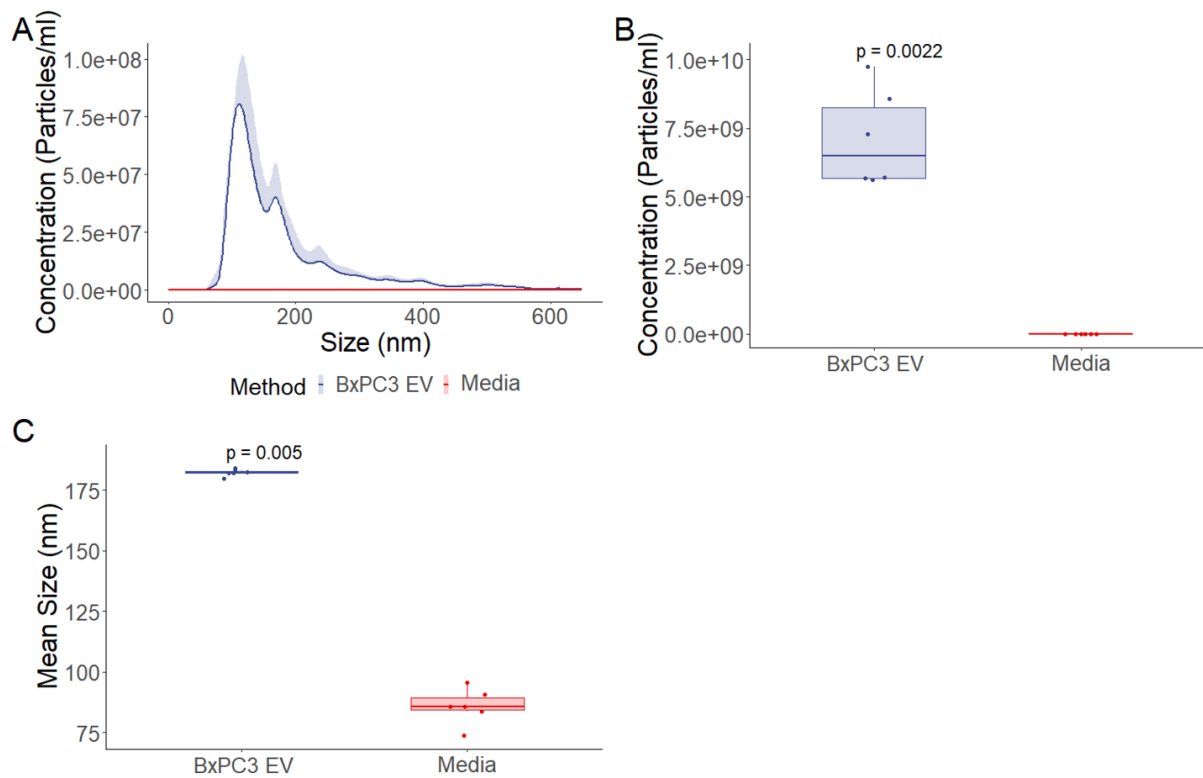


Figure 4.2. Characterisation of BxPC-3-derived EVs by NTA.

a) Nanoparticle tracking analysis (NTA) size and concentration distribution of particles in culture supernatant from serum-starved BxPC-3 cells compared to media ($n = 6$, each with 5 replicates). b) NTA particle concentration in culture supernatant from serum-starved BxPC-3 cells compared to media, ($p = 0.0022$, $n = 6$). c) NTA mean size comparison of particles in culture supernatant from serum-starved BxPC-3 cells compared to media, ($p = 0.005$, $n = 6$.)

ExoView is capable of identifying specific populations of EV based on two overlapping systems, antigenic capture with interferometric imaging and secondly fluorescent antigenic detection on the capture EV. Figure 4.3 shows ExoView interferometric imaging characterisation of EV from the culture supernatant of serum-starved BxPC-3 stratified based on capture antibody: mouse IgG control, anti-TF antibody (5G9) and anti-TF antibody (HTF-1). Figure 4.3a shows the size and concentration distribution of EV derived from the culture supernatant of serum-starved BxPC-3 cells stratified based on capture antibody. Figure 4.3b shows that 5G9 captures more ($p = 0.011$) EV compared to mouse IgG (1.8×10^8 particles/ml vs 0 particles/ml, $p = 0.011$) and that HTF-1 captures more ($p = 0.02$) EV than mouse IgG (9.1×10^7 particles/ml vs 0 particles/ml). Figure 4.3c shows that the size of EV captured on 5G9 is larger than those captured on an isotype control (68.7 nm vs 0 nm, $p = < 0.001$). HTF-1 capture EV size is also larger than Mouse IgG captured EV (62.3 nm vs 0 nm, $p = < 0.001$). Having shown that TF+ EV are captured by 5G9 and to a lesser extent with HTF-1, I then utilised the validated TF(IIID8)-AF647 shown in Figure 4.1 to stain the captured EV.

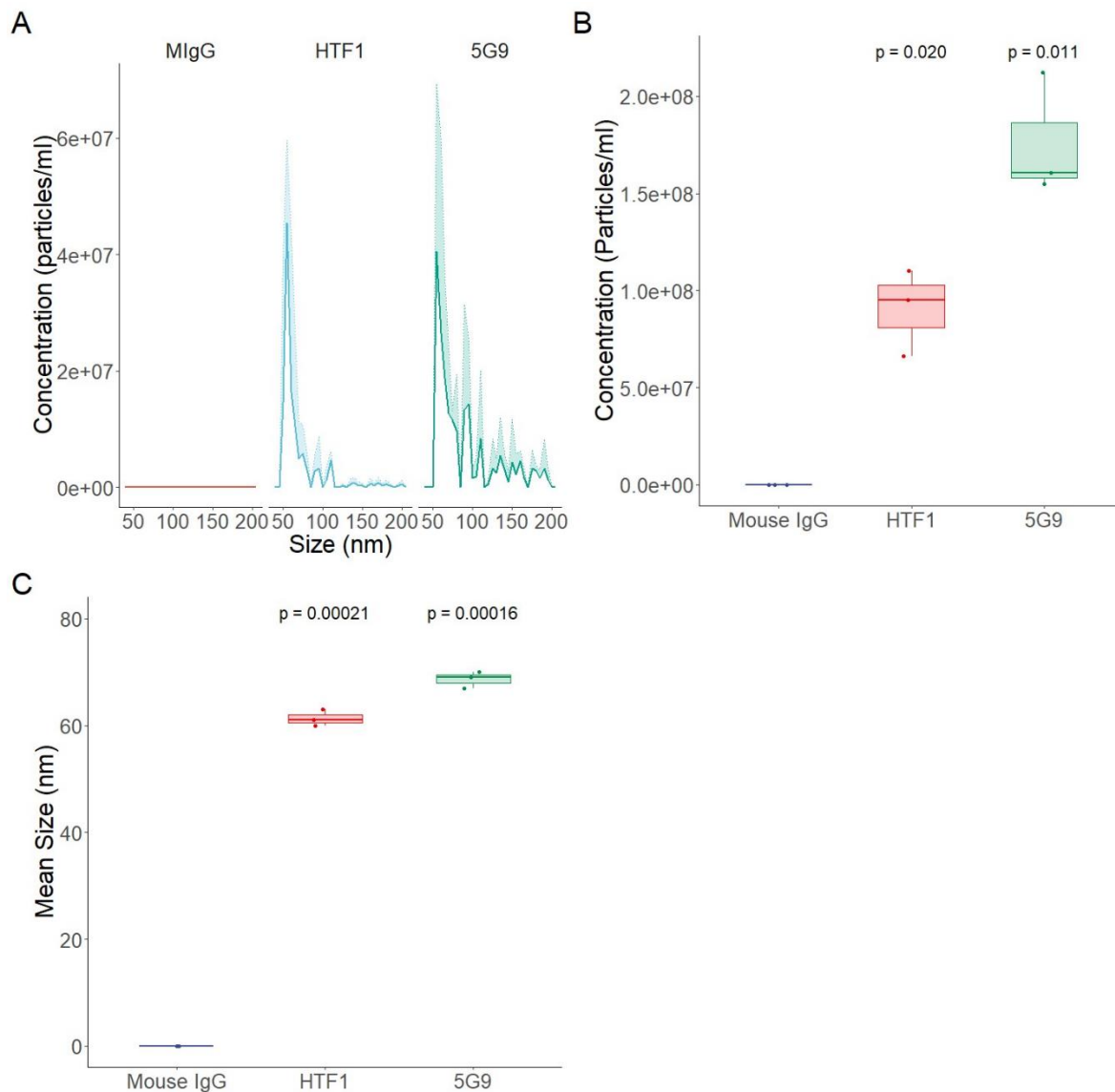


Figure 4.3. TF+ BxPC-3-derived EVs captured by ExoView technology.

A) ExoView interferometric imaging size and concentration distribution of particles in culture supernatant from serum-starved BxPC-3 cells stratified by capture marker: mouse IgG control, anti-tissue factor (TF) antibody (5G9) (n = 3, each with 3 replicates). B) ExoView interferometric imaging concentration of particles in culture supernatant from serum-starved BxPC-3 cells stratified by capture marker compared to mouse IgG capture, 5G9 capture ($p = 0.011$, n = 3). C) ExoView interferometric imaging size of particles in culture supernatant from serum-starved BxPC-3 cells stratified by capture marker compared to mouse IgG capture, 5G9 capture ($p = 0.00016$, n = 3.)

Figure 4.4 shows TF(IIID8)-AF647 fluorescent labelling of EV derived from the culture supernatant of serum-starved BxPC-3 cells captured by isotype control, anti-TF antibody (5G9) or anti-TF antibody (HTF-1). Figure 4.4a shows that the concentration of TF(IIID8)-AF647 labelled EV captured by 5G9 is significantly higher than on isotype control capture (5.3×10^6 particles/ml vs 0 particles/ml, $p = 0.0039$). Figure 4.4a also shows higher TF(IIID8)-AF647 labelled EV capture on HTF-1 compared to Mouse IgG (3.2×10^6 particles/ml vs 0 particles/ml, $p = 0.0188$). Figure 4.4b shows representative images of TF(IIID8)-AF647 fluorescent labelling of BxPC-3 derived EV stratified by antibody capture spot.

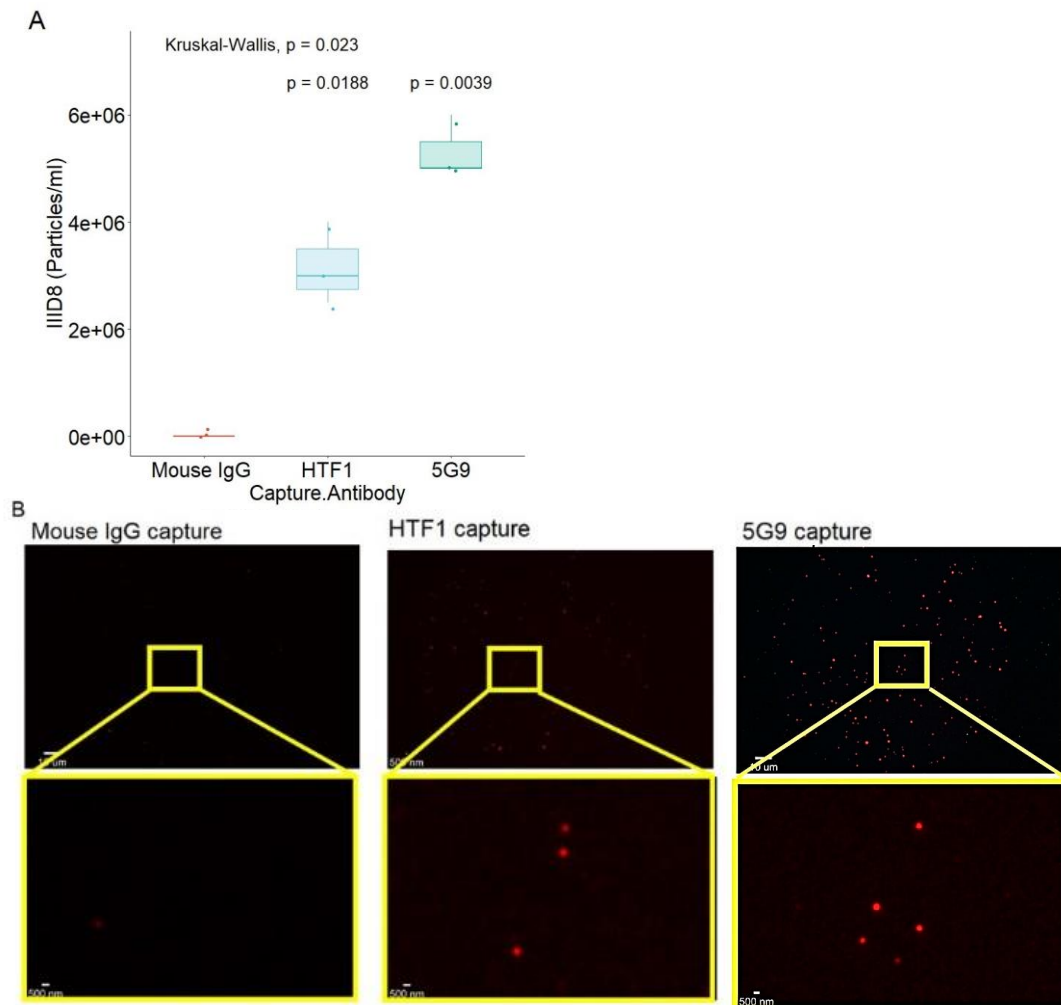


Figure 4.4. TF+ BxPC-3-derived EVs captured and fluorescently labelled by ExoView technology.

a) ExoView fluorescent labelling (anti-tissue factor (TF) antibody: IIID8-AF647) and quantification of particles in culture supernatant from serum-starved BxPC-3 cells stratified by capture marker: mouse IgG control and anti-TF antibody (5G9) ($n = 3$, each with 3 replicates). b) Representative images of TF (IIID8-AF647) labelled particles captured on TF capture spot (5G9) and control (Mouse IgG).

4.3.3 TF activity and ExoView TF detection

Cell culture supernatant is a simpler biofluid than plasma and having now established proof of principle that TF detection and capture is possible with ExoView technology, I next sought to assess whether plasma measurement was possible using positive and negative plasma samples derived from whole blood stimulated with LPS or unstimulated, respectively. For plasma EV TF detection, I first show increased concentrations of active TF from EV isolated from PFP derived from positive control LPS stimulated whole blood compared to EV isolated from PFP derived from matched negative control unstimulated whole blood (13.7 pg/ml vs 0.1 pg/ml, $p < 0.001$) (Figure 4.5a). Having shown EV TF activity in EV isolated from LPS+ PFP I next tested the thrombin generation dynamics of LPS- and LPS+ plasma measured by CAT with MP reagent. Modifying the EV TF activity assay protocol, I also double spun LPS+ plasma at 20,000 g and the plasma supernatant was run on CAT. The inhibitory anti-TF antibody HTF-1 was used on LPS+ plasma as an additional control to demonstrate that thrombin generation was TF dependent. Figure 4.5b shows median and 95% confidence intervals of thrombin generation for LPS-, LPS+, LPS+ spun and LPS+ HTF-1 over time measured by CAT. LPS stimulated whole blood derived PFP treated with isotype shows reduced lag time (Figure 4.5c) compared to matched PFP derived from unstimulated whole blood (2.3 minutes vs 6.2 minutes, $p = 0.012$). Time to peak

(Figure 4.5d) was also reduced from 7.4 minutes to 3.6 minutes in PFP derived from LPS stimulated whole blood ($p=0.012$).

After double centrifugation LPS+ plasma at 20,000 g (LPS+ spun) there was no difference compared to LPS- lag time (5.4 minutes, vs 6.2 minutes, $p = 0.6$) and time to peak (6.7 minutes vs 7.4 minutes, $p = 0.69$)

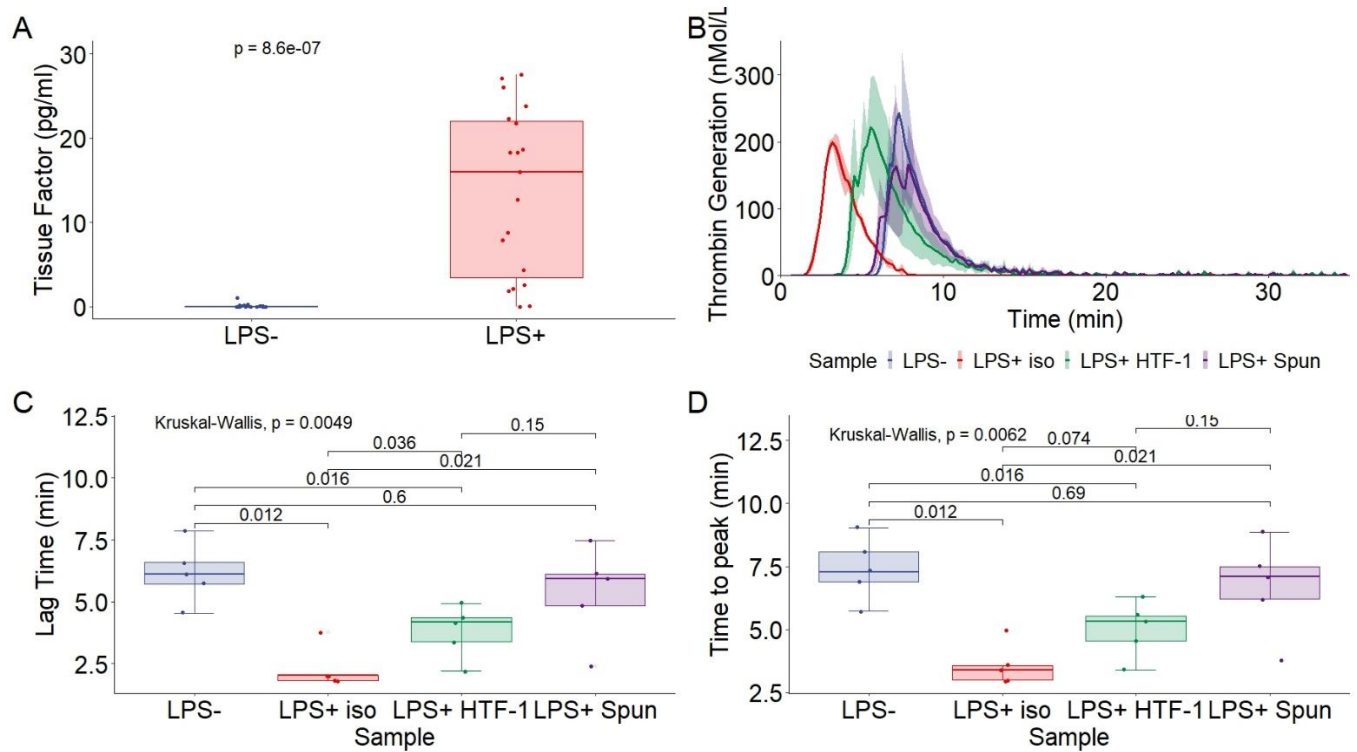


Figure 4.5. TF activity is EV associated.

A) Active tissue factor (TF) in platelet-free plasma (PFP) derived from stimulated whole blood (lipopolysaccharide (LPS+)) compared to unstimulated whole blood (LPS-) using in-house assay ($n = 19$, $p = 0.00000086$). B-D) Calibrated automated thrombography (CAT) data with microparticle reagent on LPS- PFP, LPS+ PFP and LPS+ PFP that underwent double centrifugation at 20,000 g (LPS+ spun). B) Aggregated median line graphs with confidence interval shown. C) Lag time (min), global p value = 0.0049. D) Time to peak (min), global p value = 0.0062.

Having established the presence of TF+ EV in plasma, I next sought to measure them antigenically, directly from PFP using ExoView. Figure 4.6, 4.7 and 4.8 show TF(IIID8)-AF647, CD14-FITC and CD81-AF555 fluorescent labelling of EV from LPS- and LPS+ plasma captured by antibodies against one of: CD81, CD63, CD9, CD41, TF (5G9), TF (HTF1) or Mouse IgG. An alternative visualisation of data showing all three fluorescent markers concentration per capture antibody is shown in Figure 4.9.

Figure 4.6a shows a higher concentration of CD81 captured TF(IIID8)-AF647 labelled EV in LPS+ plasma compared to LPS- plasma (4.1×10^6 particles/ml vs 1.6×10^7 particles/ml, $p = 0.016$). Figure 4.6e shows a higher concentration of 5G9 captured, TF(IIID8)-AF647 labelled EV in LPS+ plasma compared to LPS- plasma (4.6×10^7 particles/ml vs 1.2×10^7 particles/ml, $p = 0.023$). TF(IIID8)-AF647 labelled EV captured on CD63 (Figure 4.6b), CD9 (Figure 4.6c), CD41 (Figure 4.6d) TF (HTF-1) (Figure 4.6f) and Mouse IgG (Figure 4.6h) show no significant differences between LPS- and LPS+ plasma.

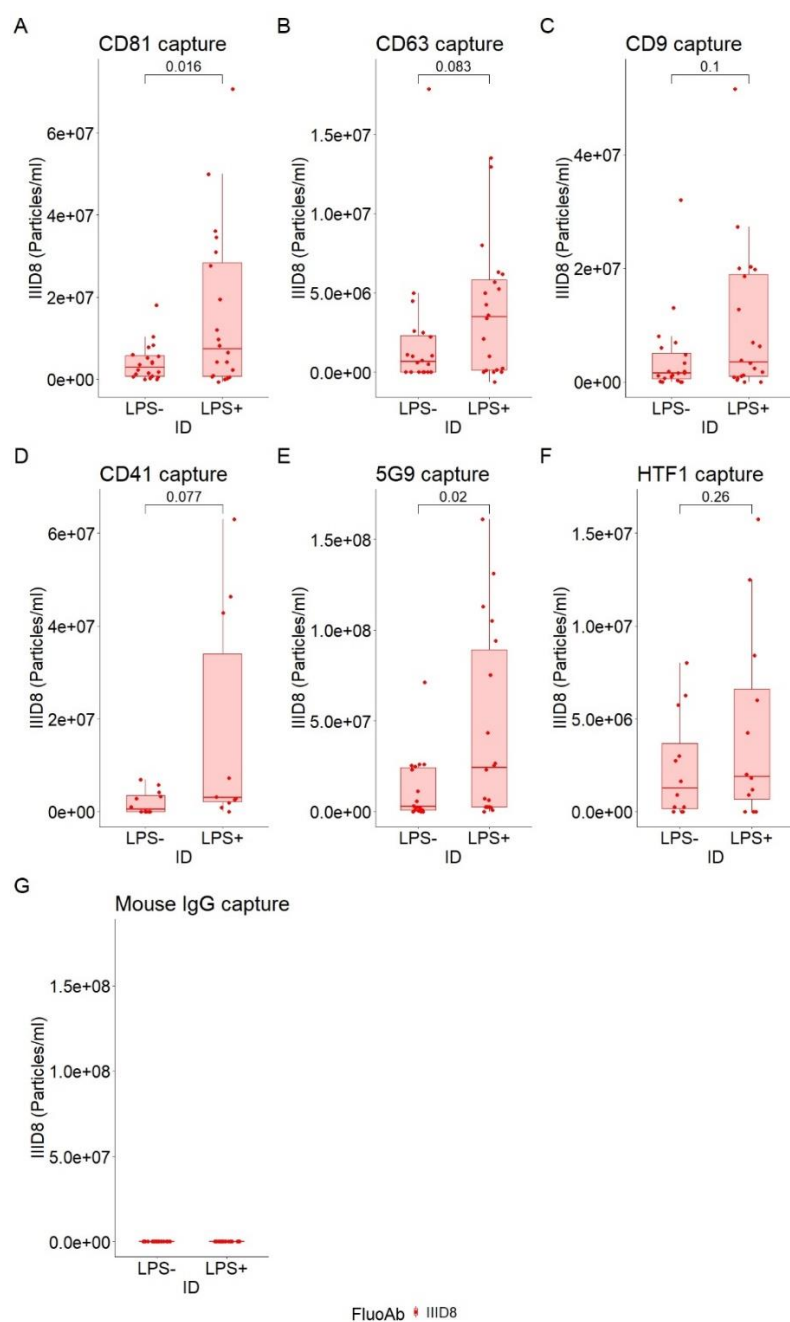


Figure 4.6. Detection of TF positive EV in platelet free plasma from LPS stimulated blood.

ExoView fluorescent extracellular vesicle (EV) count (anti-tissue factor (TF) antibody (IIID8-AF647)) on platelet-free plasma (PFP) derived from stimulated whole blood (lipopolysaccharide (LPS)+) compared to unstimulated whole blood (LPS-) with results stratified by capture marker: CD81 ($p = 0.016$, $n = 20$). (A), CD63 (B), CD9 (C), CD41a (D), anti-TF antibody 5G9 ($p = 0.02$, $n = 18$). (E), anti-TF antibody HTF-1 (F) and Mouse IgG control (G).

Figure 4.7 shows CD14-FITC fluorescent labelling of EV from LPS- and LPS+ plasma captured by antibodies against one of: CD81, CD63, CD9, CD41, TF (5G9), TF (HTF1) or Mouse IgG. Figure 4.7d shows a higher concentration of CD41 captured CD14-FITC labelled EV in LPS+ plasma compared to LPS- plasma (8.6×10^7 particles/ml vs 8.1×10^6 particles/ml, $p = 0.023$). CD14-FITC labelled EV captured on CD81 (Figure 4.7a), CD63 (Figure 4.7b), CD9 (Figure 4.7c) TF (5G9) (Figure 4.7e), TF (HTF-1) (Figure 4.7f) and Mouse IgG (Figure 4.7g) show no significant differences between LPS- and LPS+ plasma.

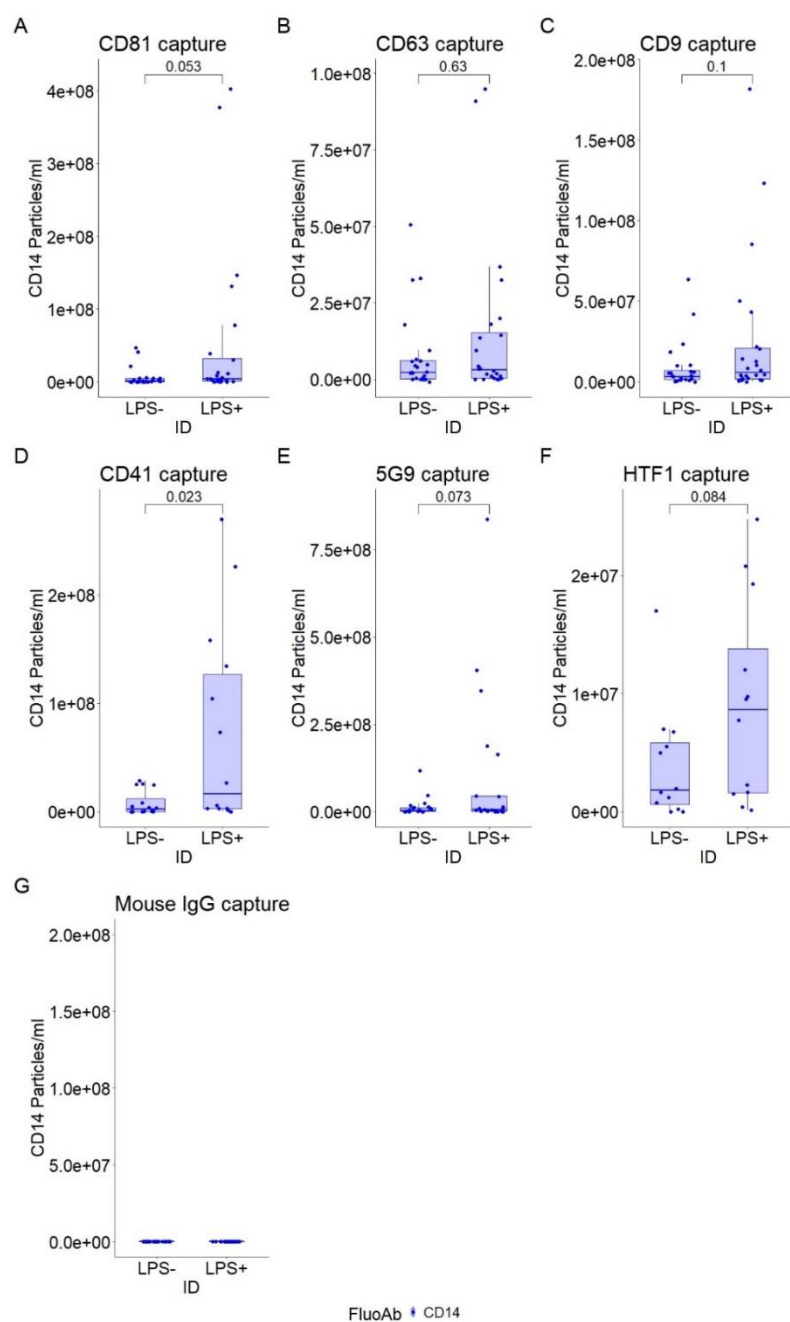


Figure 4.7. Detection of CD14 positive EV in platelet free plasma from LPS stimulated blood.

ExoView fluorescent extracellular vesicle (EV) count (anti-CD14-FITC) on platelet-free plasma (PFP) derived from stimulated whole blood (lipopolysaccharide (LPS)+) compared to unstimulated whole blood (LPS-) with results stratified by capture marker: CD81 (A), CD63 (B), CD9 (C), CD41a (p = 0.023, n = 15) (D), anti-TF antibody 5G9 (E), anti-TF antibody HTF-1 (F) and Mouse IgG control (G).

Figure 4.8 shows CD63-PE fluorescent labelling of EV from LPS- and LPS+ plasma captured by antibodies against one of: CD81, CD63, CD9, CD41, TF (5G9), TF (HTF1) or Mouse IgG. Figure 4.8d shows a higher concentration of CD41 captured CD63-PE labelled EV in LPS+ plasma compared to LPS- plasma ($p = 0.0087$). Figure 4.8e shows a higher concentration of 5G9 captured CD63-PE labelled EV in LPS+ plasma compared to LPS- plasma ($p = 0.016$). CD63-PE labelled EV captured on CD81 (Figure 4.8a), CD63 (Figure 4.8b), CD9 (Figure 4.8c), TF (HTF-1) (Figure 4.8f) and Mouse IgG (Figure 4.8g) show no significant differences between LPS- and LPS+ plasma. Having established that 5G9 capture combined with IIID8-AF647 labelling shows increased concentration of EV in LPS+ samples I next wanted to determine if ExoView antigenic detection could detect TF+ EV in clinical samples. For this purpose, a trauma cohort and an ovarian cancer cohort were used.

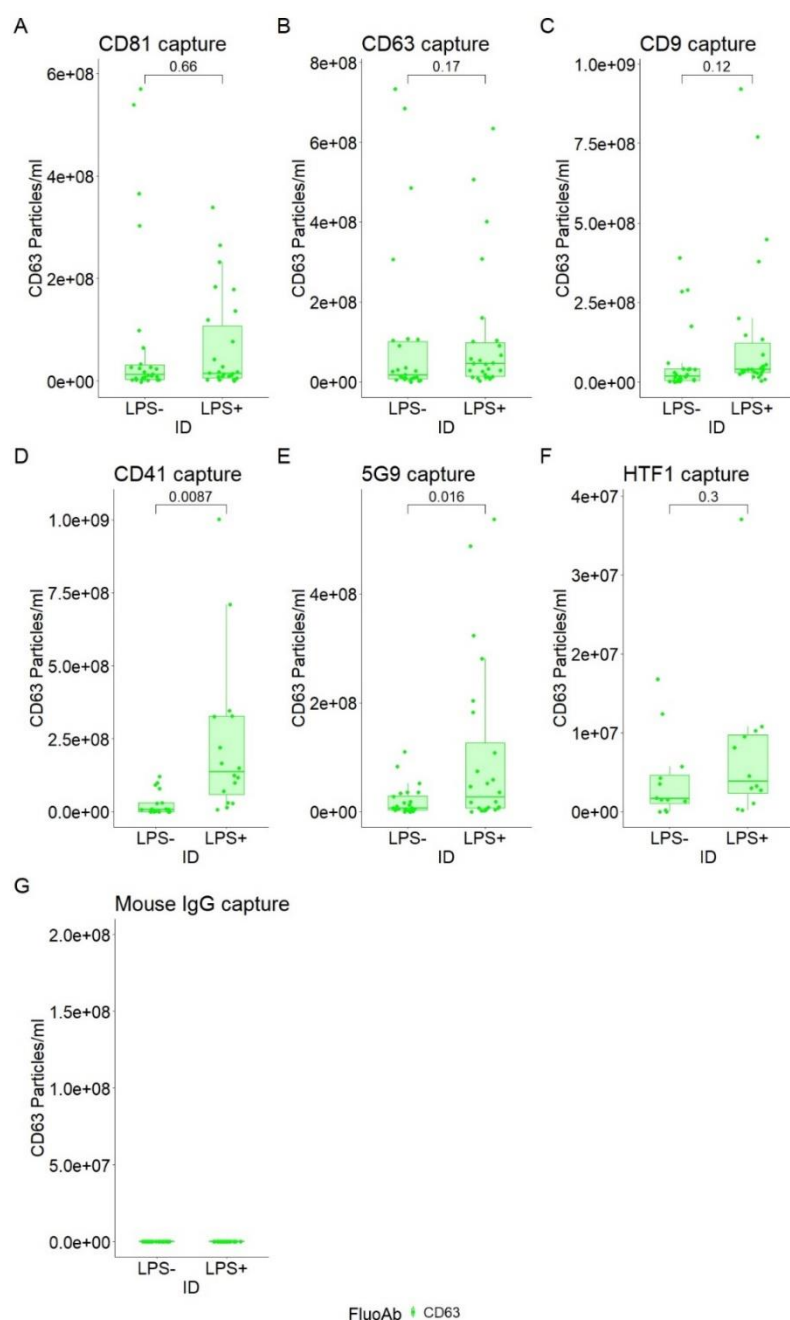


Figure 4.8. Detection of CD63 positive EV in platelet free plasma from LPS stimulated blood.

ExoView fluorescent extracellular vesicle (EV) count (anti-CD63-PE) on platelet-free plasma (PFP) derived from stimulated whole blood (lipopolysaccharide (LPS)+) compared to unstimulated whole blood (LPS-) with results stratified by capture marker: CD81 (A), CD63 (B), CD9 (C), CD41a (p = 0.0087, n = 15) (D), anti-TF antibody 5G9 (p = 0.016, n = 21) (E), anti-TF antibody HTF-1 (F) and Mouse IgG control (G).

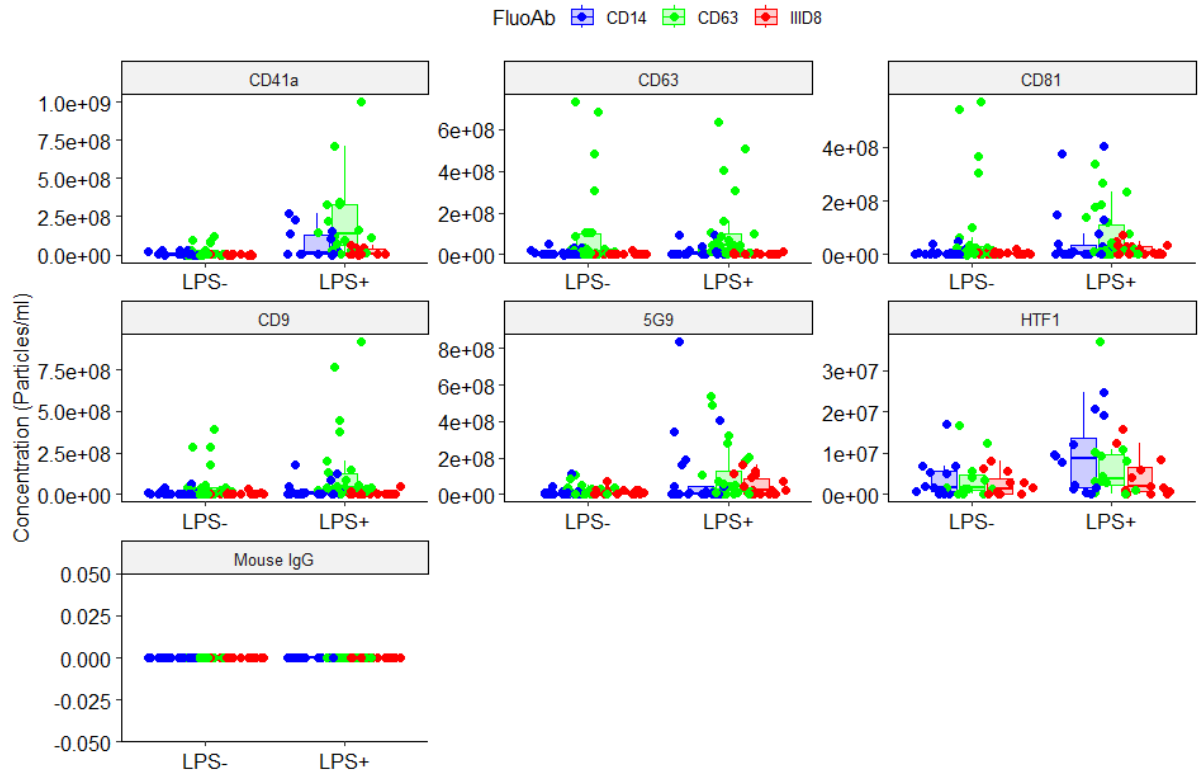


Figure 4.9. Alternative visualisation of secondary labelling of platelet free plasma from LPS stimulated blood.

ExoView fluorescent extracellular vesicle (EV) count (anti-CD14-FITC, anti-CD63-PE, anti-tissue factor (TF) (IIID8-AF647)) on platelet-free plasma (PFP) derived from stimulated whole blood (lipopolysaccharide (LPS)+) compared to unstimulated whole blood (LPS-) with results stratified by capture marker: CD41a, CD63, CD81, CD9, anti-TF antibody 5G9, anti-TF antibody HTF-1 and Mouse IgG control

4.3.4 Measurement of TF-EV within pathological samples

Figure 4.10a shows increased concentrations of active TF on EVs isolated from PFP derived from trauma patients' PFP (0.940 pg/ml, $p = 0.0363$) taken within 1 hour of injury and ovarian cancer patient's samples PFP (0.363 pg/ml, $p = 0.0037$) compared to healthy control PFP (0.089 pg/ml). Figure 4.10b shows that the concentrations of TF(IIID8)-AF647 labelled EV captured on isotype are not significantly different

between healthy (n = 18), trauma (n = 8) and ovarian cancer PFP (n = 14). Similarly, Figure 4.10c shows that the concentrations of 5G9 captured, TF(IIID8)-AF647 labelled EV are not significantly different between healthy, trauma and ovarian cancer PFP. Figure 4.10d shows representative images of TF(IIID8)-AF647 fluorescent labelling of EV in healthy trauma and ovarian cancer PFP, stratified by antibody capture spot. These representative images show some TF positivity, but the overall data was not significant.

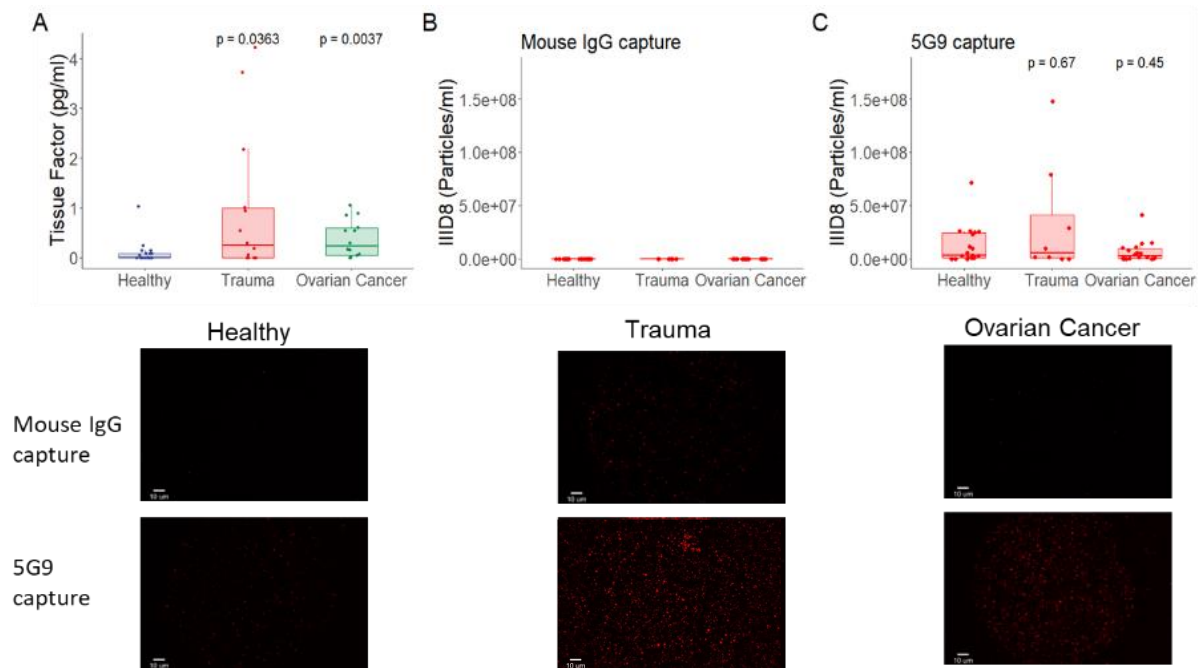


Figure 4.10. TF activity and ExoView measurement in trauma and ovarian cancer.

A) Active extracellular vesicle (EV) tissue factor (TF) in platelet-free plasma (PFP) derived from trauma patients within 1 hour of injury ($p = 0.0363$, $n = 14$) and ovarian cancer patients ($p = 0.0037$, $n = 13$) compared against healthy controls, using an in-house assay B-C) ExoView fluorescent extracellular vesicle (EV) count (anti-TF antibody (IIID8-AF647)) on PFP derived from healthy controls ($n = 18$) trauma patients within 1 hour of injury ($p = 0.67$, $n = 8$) and ovarian cancer patients ($p = 0.47$, $n = 13$) with results stratified by capture marker, results are compared to healthy controls B) mouse IgG control, C) anti-TF antibody (5G9) D) Representative images of anti-TF antibody (IIID8-AF647) staining stratified by capture marker: mouse IgG control and anti-TF antibody (5G9)

4.3.5 Validation of ExoView EV TF measurements

Figure 4.11 shows correlations between antigenic fluorescent TF+ EV measurements made by ExoView between different TF antibody capture spots compared to TF activity. Figure 4.11a shows that there is a positive relationship between 5G9 captured TF(IIID8)-AF647 labelled EV and TF activity in LPS- and LPS+ PFP ($R^2 = 0.28$, $p = 0.011$). Figure 4.11b and 4.11c show no significant association between 5G9 captured TF(IIID8)-AF647 in PFP from patients with trauma (Figure 4.11b) or ovarian cancer (Figure 4.11c).

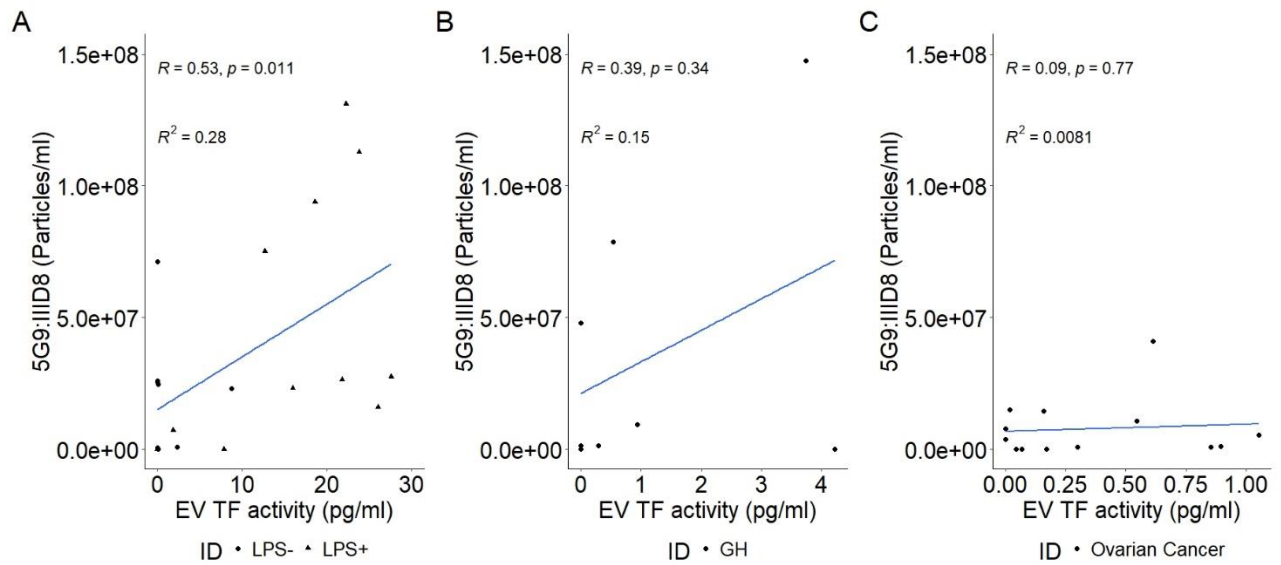


Figure 4.11. TF correlations between ExoView measurements and TF activity assay.

A) 5G9 captured IIID8 labelled extracellular vesicle (EV) counts relationship with EV tissue factor (TF) activity ($R^2=0.28$, $p = 0.011$, $n = 22$ (11 lipopolysaccharide (LPS)-, 11 LPS+)). B) 5G9 captured IIID8 labelled EV counts relationship with EV TF activity in trauma patients ($R^2 = 0.15$, $p = 0.34$, $n = 8$). C) 5G9 captured IIID8 labelled EV counts relationship with EV TF activity in ovarian cancer patients ($R^2 = 0.0081$, $p = 0.77$, $n = 13$).

4.4 Discussion

The ExoView R100 system has been utilised for characterising EV in health, disease and supernatants from cell culture [70, 309, 310, 357]. The vast majority of the literature on ExoView focuses on capture and detection of EV based on tetraspanin expression, such as CD63, CD9 and CD81 [309]. There are some novel uses of ExoView in identifying markers of interest, for instance, following tetraspanin capture, CD14 has been shown to be a marker of acute respiratory distress syndrome [358]. Antigenically measuring TF+ EV in plasma has been historically challenging; the low concentration of TF+ EV in plasma often falls under the detection limit of conventional antigenic assays [207, 217-219]. Furthermore, flow cytometry is often incapable of resolving small EV [295]. TF measurement is also lacking a gold-standard assay and a well-defined, reproducible standard. Several TF ELISAs have been developed and in some cases compared against one another, normally with non-plasma samples [322, 359] but they still often fail to detect TF in plasma [73, 220]. Despite these issues, roadmaps have been built to pave the way towards optimising antigenic TF EV detection in plasma [214, 215]. I hypothesised that the most promising sandwich ELISA combinations combined with ExoView technology could therefore allow for direct capture and quantification of TF EV [322, 359].

I first tested IIID8-AF647, VIC12-AF647, and 5G9-AF647 by monocyte flow cytometry. These data showed a significant increase in TF expression and MFI after 5 hours of LPS stimulation with all 3 antibodies. As with previous studies, there are high and low responders to LPS stimulation [203, 360]. In contrast to the existing literature, some of the unstimulated monocytes appear to be expressing more background TF than

typically expected. Normal ranges for unstimulated monocytes are usually typically under 1.5%; however, our data show that 21.3% (IIID8), 9.5% (VIC12), and 21.8% (5G9) of monocytes express TF. The specificity of both IIID8 and 5G9 antibodies has been determined before [74, 325, 361]. It is possible that time delays in processing blood for monocyte TF measurements may have contributed to the increased expression in TF in some of the samples. But it is important to state that the LPS- control PFP samples for ExoView and TF⁺ EV samples were processed immediately without any delays to minimize any potential induction of TF expression by monocytes and release of TF⁺ EV. IIID8-AF647 showed the greatest MFI difference between LPS- and LPS+ samples and was, therefore, chosen for fluorescent detection with ExoView.

Using thrombin generation by CAT there is TF activity associated with EV in the absence of cells. In line with previous studies [362], that lag time and time to peak are significantly reduced in LPS+ PFP compared to LPS- PFP. I also show that pre-treatment of LPS+ plasma with an inhibitory anti-TF antibody (HTF-1) partially ablates the reductions in lag time and time to peak. Furthermore, the removal of EV through double centrifugation at 20,000 g also fully reverses the reduced lag time and time to peak of LPS+ samples. These findings indicate that TF activity in plasma is therefore EV associated rather than related to the soluble alternately spliced TF variant form [198].

The anti-TF monoclonal antibodies 5G9 and IIID8 bind to TF at residues 181-214 and towards the c-terminal of TF (residues 1-25), respectively [319]. Theoretically, only a combination of monoclonal antibodies that bind to distinct epitopes should be used for

TF detection in the form of sandwich ELISAs [322, 359]. I show that in-house conjugated IID8-AF647 binds to TF on LPS stimulated monocytes. As with previous studies there are high and low responders to LPS stimulation [323]. Combining IID8-AF647 with an anti-TF antibody 5G9 capture system on the ExoView chips resulted in successful detection of TF EV and allowed for their measurement in BxPC-3 cell culture supernatants and within the LPS-positive control PFP. To my knowledge this is the first use of ExoFlex technology to enable the customised capture and detection of a rare EV population. Despite promising LPS+ results IID8-AF647 labelling of 5G9 captured TF+ EV concentration does not increase with trauma or ovarian cancer samples using ExoView despite detected increases in TF activity measurements.

Cell culture supernatant derived from serum-starved cancer cells was shown to be a compatible biofluid for NTA analysis, however NTA was not capable of determining whether these EV expressed TF. Interestingly, in line with previous publications [29], the recorded size of EV with ExoView was considerably smaller than that of NTA. ExoView size measurements have been shown to align with transmission electron microscopy (TEM) size results, whereas NTA is subject to broader size distributions and substantially larger recorded sizes [363]. The seeming size change in particles between different measurement technologies is interesting; NTA is likely to be overestimating size due to the nature of the measurement settings and inability to resolve EV from other similarly sized particles in plasma. Furthermore, the sample is “hydrated” for NTA measurement (i.e. not dried and captured as with TEM and ExoView) [363]. However, NanoFlow Cytometry (NanoFCM) which also relies on sample being hydrated and generates size distribution profiles that are more

consistent with TEM and ExoView [364]. It may be more likely that the inherent limitation of Brownian motion-based detection methods (NTA) combined with low optical resolution are responsible for the discrepancy between NTA and other measurement modalities [363].

The LPS-/± PFP TF activity varied from 0 - 30pg/ml, over this range there was a significant association with 5G9 captured, IIID8-AF647 labelled EV concentrations. As one might expect, EV TF activity in the samples from the trauma and the ovarian cancer cohorts were significantly higher than in healthy controls. In trauma EV TF activity varied from 0 – 4 pg/ml and the association with 5G9 captured, IIID8-AF647 labelled EV concentration was not significant. Furthermore, in ovarian cancer, where EV TF activity varied from 0 - 1 pg/ml, the relationship with 5G9 captured, IIID8-AF647 labelled EV concentration was not significant. These data suggest that although antigenic TF detection of EV in plasma is possible, in the two types of clinically relevant samples tested here, it may be of limited utility. At present, the threshold for utility of antigenic TF+ EV detection in plasma by ExoView has not been established but I estimate that an association exists above 5 pg/ml of TF activity. Interestingly, there is antigenic signal measured by ExoView that clusters at 0 pg/ml, across all cohorts measured. This could indicate a failure of antigenic detection or even represent a pool of encrypted TF+ EV which exhibits no activity. Encrypted TF+ EV may represent a latent pool of potential TF activity [325].

A limitation of this chapter is the small number of clinical samples available for use across all of the assays employed. However, this work has allowed for the use of ad hoc power tests which determine that 23 trauma patients would be required to

establish whether TF activity and TF+ EV detection by ExoView are significantly related. I did investigate whether TF EV concentration and activity correlated with injury severity and TBI status but there was no relationship (data not shown). My thinking was that traumatic brain injury is more associated with hypercoagulability than orthopaedic trauma, this is believed to be due to the high expression of TF in brain tissue and released particles upon trauma [209].

It is also worth considering that other approaches may be beneficial, one publication shows effective immunomagnetic isolation of EV from plasma via CD29 and CD59 capture, these isolated EV are comparable to isolated EV by centrifugation in terms of TF activity [365].

Chapter 5. On the origin of circulating EV

5.1 Rationale

EV in the blood are heterogeneous and originate from circulating cells, from cells in contact with the vasculature (i.e. the vascular endothelium) and from the tissues [142]. The vast majority of circulating EV are derived from circulating blood cells including platelets, white blood cells and red blood cells [366]. A recent publication used RNA profiling on EV isolated from plasma and determined that 98% of circulating EV are derived from circulating blood cells with the remaining 2% from the tissues [142]. However, this paper does rely upon the use of an algorithm that is not shared and so has not been reproduced. However, many review articles refer to platelets as the major source of circulating EV with ranges varying from 1.2×10^4 EVs/ μ L to 6.3×10^9 EV/ μ L [367-369]. These ranges suggest approximately 25-75% of circulating EV are platelet-derived. However, the earliest papers that report this claim also refer to platelets and/or megakaryocytes (MK) as the major source of circulating EV in health [143, 186, 367, 370]. In practice it can be difficult to differentiate PEV and MK EV from each other. This has been, in part, due to historical measurement challenges associated with EV measurement. However, there are also a number of key problems with distinguishing PEV and MK EV from each other including their identical cell lineage and thus phenotype [371]; residual platelets in plasma samples causing PEV release during any subsequent freezing and thawing which exaggerate the concentration of PEV and that *ex vivo* platelet activation can cause PEV contamination of plasma [229, 236]. As platelets are derived from MK, specific unique markers of either PEV or MK EV have largely been elusive. Specific candidate markers can also be difficult to validate as bone marrow isolation and *in vitro* cell culture of MK are not only technically demanding

and challenging but may require iPSC reprogramming and MK differentiation, both of which may alter EV biogenesis and phenotype [372]. Furthermore, cultured MK are also heterogeneous with low numbers of cells in different stages of maturation and often contaminated with cell debris (expressing platelet/MK markers) and if terminally differentiated to induce proplatelet formation and eventual thrombopoiesis could also potentially release platelets [189]. These platelets may then release EV thereby affecting the validation of specific MK EV markers. MK EV are also thought to be released constitutively in the absence of cellular activation, whereas platelets are thought to require activation for EV release [186]. Both platelets and MK can release exosomes with common markers including CD63 and CD9. Furthermore, CD9 is highly expressed on the plasma membrane of both platelets and MK. However, despite all these issues a number of candidate markers have been proposed. A recent review highlighted a panel of specific markers that either have been shown or are theoretically unique to either platelets, MK and/or their derived EV [187]. This review claims that GPVI should be MK EV specific, CLEC2 should be expressed on both PEV and MK EV and finally LAMP-1 is theoretically PEV specific [187]. According to this review, GPVI is cleaved upon platelet activation and it is suggested that PEV are released upon activation and therefore GPVI should be absent, whereas constitutive EV release from MK will not be preceded by GPVI cleavage. CLEC2 does not undergo cleavage events and is thought to be expressed on both PEV and MK EV. LAMP 1 is a platelet activation marker that should be absent on unstimulated MK and their EV but is theoretically present on PEV. Deciphering the relative contribution of platelet and MK to circulating EV pools may reveal new insights into MK and platelet biology which may have implications for biomarkers of CVD and understanding homeostatic

mechanisms that maintain health [186]. The Italiano group suggest in health the vast majority of EV are MK derived and a shift towards platelet EV is indicative of platelet activation in the circulation for example in CVD [186]. However, the Italiano group that suggested this hypothesis have not produced any outputs on this topic for over a decade. The thinking that GPVI cleavage and platelet activation is essential for PEV release may only hold for sustained platelet activation and fails to take into account basal EV biogenesis of PEV which may not include GPVI cleavage events.

Platelets are often a source of artefactual EV released upon freeze-thawing of plasma samples that contain residual platelets [237]. Plasma samples are often described as either PPP or PFP depending on the concentration of residual platelets which is normally determined by the number of centrifugation steps used in the preparation [236]. For EV studies, PFP prepared by at least a double centrifugation protocol has been recommended by ISTH and ISEV guidelines due to the potential contribution of EV and EV-like material released during freeze thawing of residual platelets [236, 237]. However, a question has also recently been raised about whether PFP is completely platelet free [238].

Some authors have recently demonstrated that residual platelets still remain following double centrifugation at a concentration undetectable by conventional whole blood analysers but detectable by more sensitive techniques such as flow cytometry [237]. Two papers have recently shown that filtration through a 0.8 μm filter further reduces platelet contamination [237, 238]. Bettin *et al* suggest filtering will reduce PEV contamination in freeze-thawed samples despite showing no difference in CD61 (platelet/MK specific marker) positive EV before and after filtration [237]. A good place

to start when trying to identify whether PEV or MK EV are the dominant circulating EV population is by validating prospective markers of origin in plasma, MK and platelet supernatants. Knowing that PPP contains residual platelets which result in artefactual PEV increase means that a comparison of PPP and PFP EV may reveal a PEV specific signature. Platelets are fairly straightforward to isolate and activate, which means they form an accessible biofluid to test EV markers of platelet specific origin in the absence of other cells and their derived EV. The markers mentioned above, GPVI, CLEC2 and LAMP1 coupled with tetraspanin plasma chip capture by ExoView may therefore allow for distinction of MK EV from PEV. This is currently difficult to do on any other platforms due to the low number of lasers and fluorescent emission detectors available on nanoflow cytometers and inability of most platforms to resolve small EV [373].

5.2 Aims

- Establish whether there are platelet or/and MK specific EV markers in plasma.
- Measure MK and platelet EV specific markers in plasma and platelet and megakaryocyte derived supernatants.

5.3 Results

5.3.1 Platelet and EV characterisation of PPP and PFP

To start with, a comparison of PPP and PFP was conducted. The literature suggests that residual platelet concentration in PPP is sufficient to induce artefactual platelet EV generation upon freeze-thawing [237]. Whole blood cell counting was performed on donor matched PPP and PFP (n = 13) prior to freezing. As expected, PPP contained a higher concentration of platelets than PFP ($9 \times 10^9/L$ vs $0 \times 10^9/L$, p =

0.005) (Figure 5.1). The impedance counts provided by the full blood counter (Sysmex XN1000) for platelet counts are expressed as “ $\times 10^9/L$ ” (equivalent to $\times 10^6/ml$ or $\times 10^3/\mu l$), values less than $1 \times 10^9/L$ will therefore appear as 0 but the range between $0-1 \times 10^9/L$ is still theoretically large and therefore PFP may still contain significant amounts of residual platelets which remain a significant potential confounder for accurate PEV and MK EV biomarker measurements. For this reason, an experiment was then conducted comparing PFP with PFP that had also been $0.8 \mu m$ filtered. A smaller $0.22 \mu m$ filter was also used as a positive control to fully maximise platelet removal. Unsurprisingly, this also confirmed PFP to be platelet-free. However, due to the limit of detection of the full blood counter these samples were also counted by flow cytometry using CD61-FITC labelling with a direct volume counting method (see section 2.1.15 Platelet flow cytometry) [331]. Flow cytometric platelet counts demonstrated that PFP still contained 0.27×10^9 platelets/L which was significantly greater than filtered PFP using either $0.8 \mu m$ (0.041×10^9 platelets/L, $p = 0.0022$) or $0.22 \mu m$ (0.0131×10^9 platelets/L, $p = 0.005$) pore size filters with no significant difference ($p = 0.52$) between the two filters (Figure 5.1).

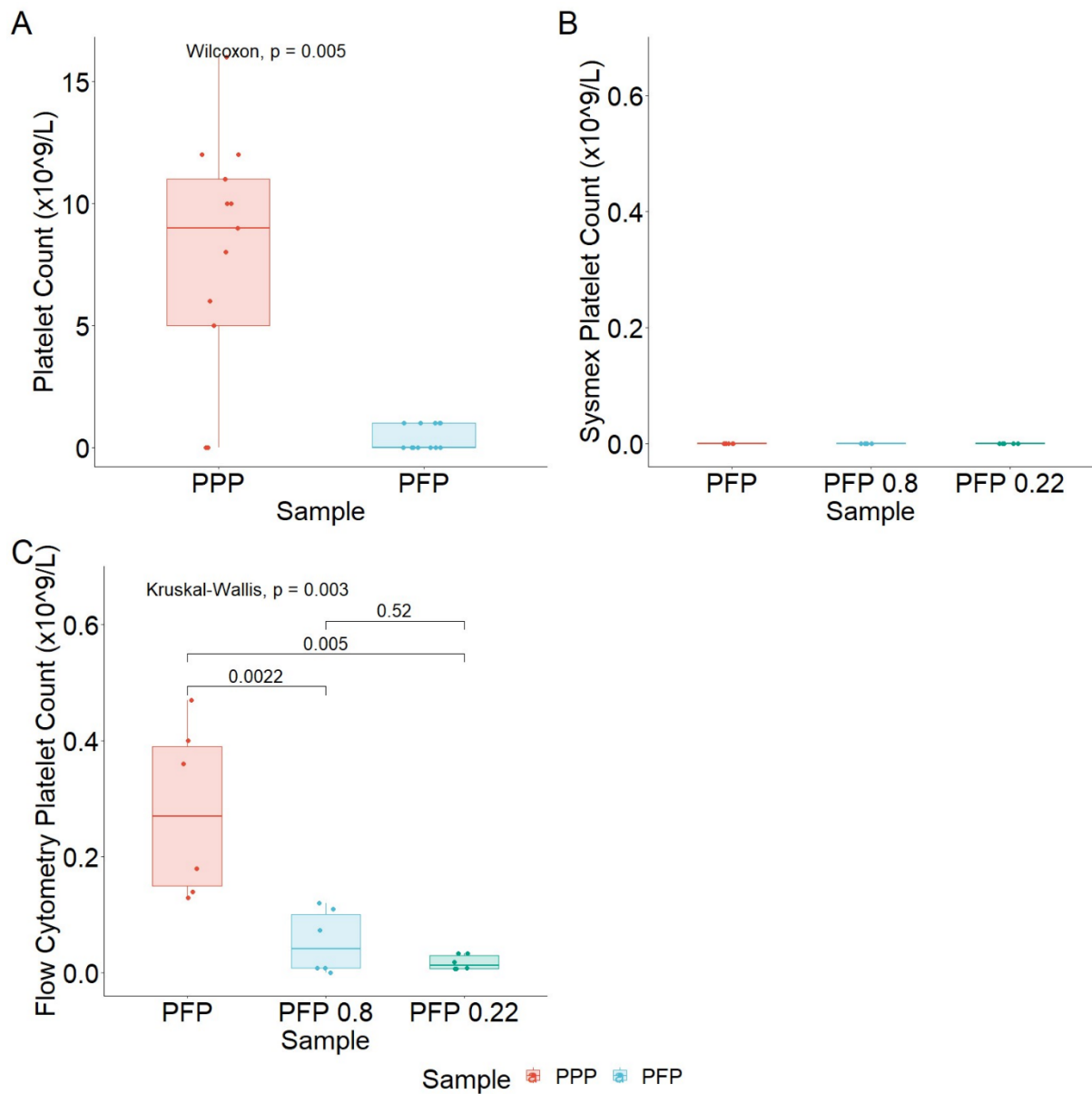


Figure 5.1. Platelet counts in PPP and PFP.

a) Platelet poor plasma (PPP) and platelet free plasma (PFP) impedance platelet counts (Sysmex XN-1000) ($p = 0.005$, $n = 13$) b) PFP and filtered PFP (0.8 μm or 0.22 μm filters) impedance platelet counts (Sysmex XN-1000) ($n = 6$) c) PFP and filtered PFP (0.8 μm or 0.22 μm cut-off filters) platelet counts (flow cytometry) (global $p = 0.003$, $n = 6$)

The effect of residual platelets in plasma has previously been demonstrated to increase the generation of platelet derived EV either by activation in fresh plasma or as a consequence of freeze thawing after storage [229, 236]. Next, I therefore sought to confirm whether the reduction in platelet contamination between PPP and PFP shown above could also translate to a reduction in PEV after freeze/thawing with ExoView. This experiment utilised standard tetraspanin plasma chips and standard fluorescent antibodies (CD9-AF488, CD81-AF555, CD63-AF647). Interferometric imaging concentration data is shown in Figure 5.2 for PPP compared to PFP. Levels of CD81, CD63 and CD9 EV showed no significant differences. However, the concentration of CD41 captured EV was significantly reduced in PFP (8.0×10^7 particles/ml) compared to PPP (1.2×10^8 particles/ml, $p = 0.016$). Remnant platelets in PPP therefore contribute to an increased PEV count by antigenic ExoView detection in freeze-thawed plasma.

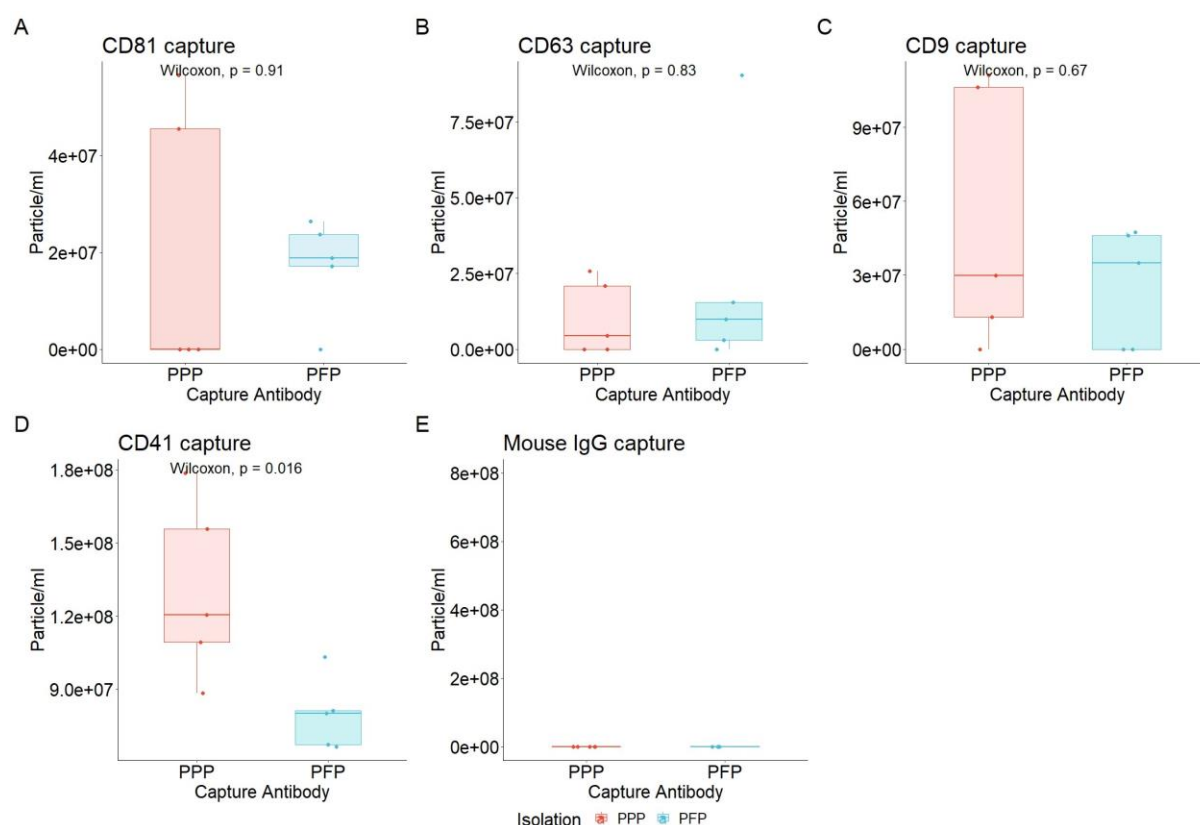


Figure 5.2. Extracellular vesicle (EV) concentrations in PPP and PFP.

a) ExoView EV interferometric imaging concentration in PPP and PFP with results stratified by capture marker: a) CD81 b) CD63 c) CD9 d) CD41a ($p = 0.016$) and e) Mouse IgG control, $n = 5$.

With secondary fluorescent labelling of captured EV in PPP and PFP expression of CD81, CD63 and CD9 were also measured. Figure 5.3 shows no significant changes in CD81 captured EV between PPP and PFP when measured by secondary labelling with either CD81, CD63 or CD9. However, there was a reduction in CD41 captured, CD81 labelled EV from PPP (1.2×10^8 particles/ml) to PFP (7.3×10^6 particles/ml, $p = 0.0079$). Figure 5.4 shows no significant differences of CD63 captured EV when measured by secondary labelling across all capture spots. Figure 5.5 shows no significant differences between CD9 captured EV when measured by secondary labelling. For the remaining capture spots, however, there were significant decreases in levels of CD9 captured EV between PPP and PFP, with CD81 (8.7×10^7 vs 1.5×10^7 , $p = 0.032$), CD9 (3.8×10^8 vs 4.5×10^7 , $p = 0.016$) and CD41 capture (2.5×10^8 vs 2.6×10^7 , $p = 0.016$).

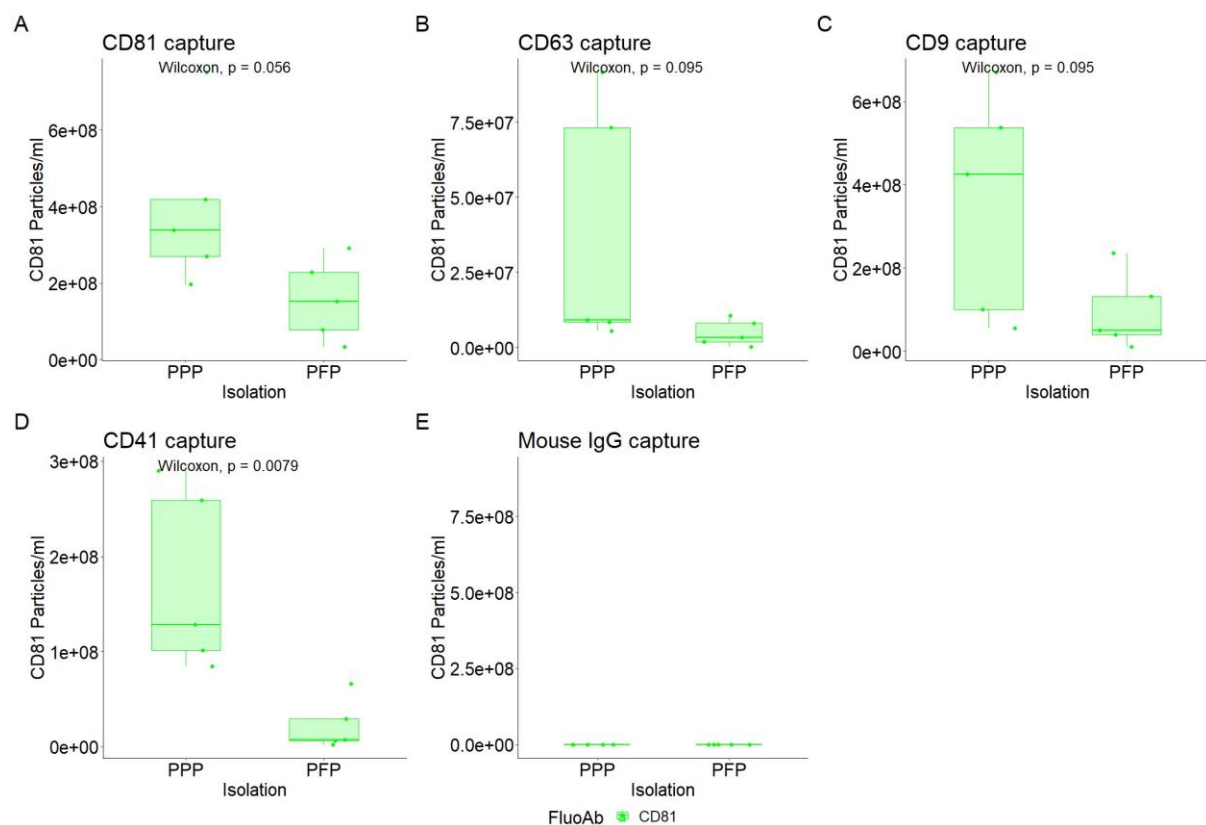


Figure 5.3. Detection of CD81 positive extracellular vesicles (EV) in platelet poor plasma (PPP) and platelet free plasma (PFP).

ExoView fluorescent EV count (anti-CD81-AF555) on PPP and PFP with results stratified by capture marker: a) CD81 b) CD63 c) CD9 d) CD41a ($p = 0.0079$) e) Mouse IgG control, $n = 5$.

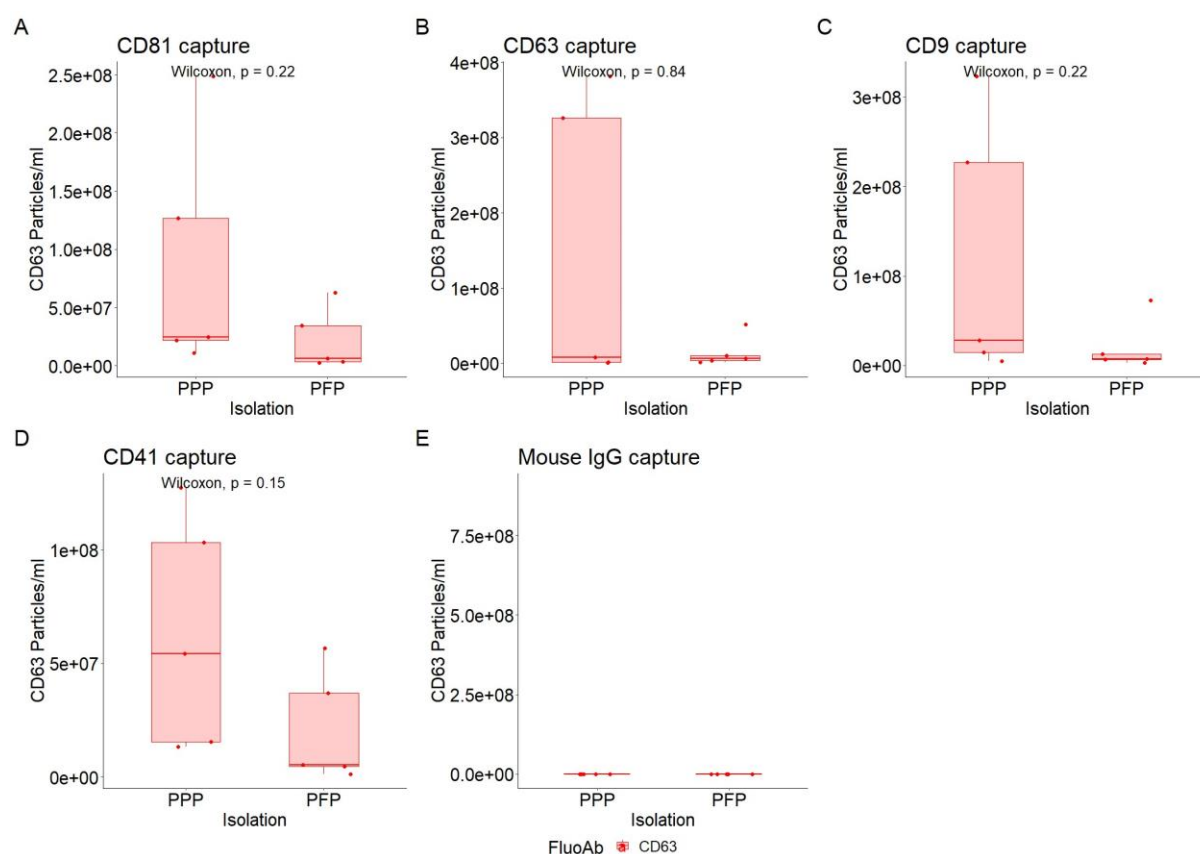


Figure 5.4. Detection of CD63 positive extracellular vesicles (EV) in platelet poor plasma (PPP) and platelet free plasma (PFP).

ExoView fluorescent EV count (anti-CD63-AF647) on PPP and PFP with results stratified by capture marker: a) CD81 b) CD63 c) CD9 d) CD41a e) Mouse IgG control, $n = 5$.

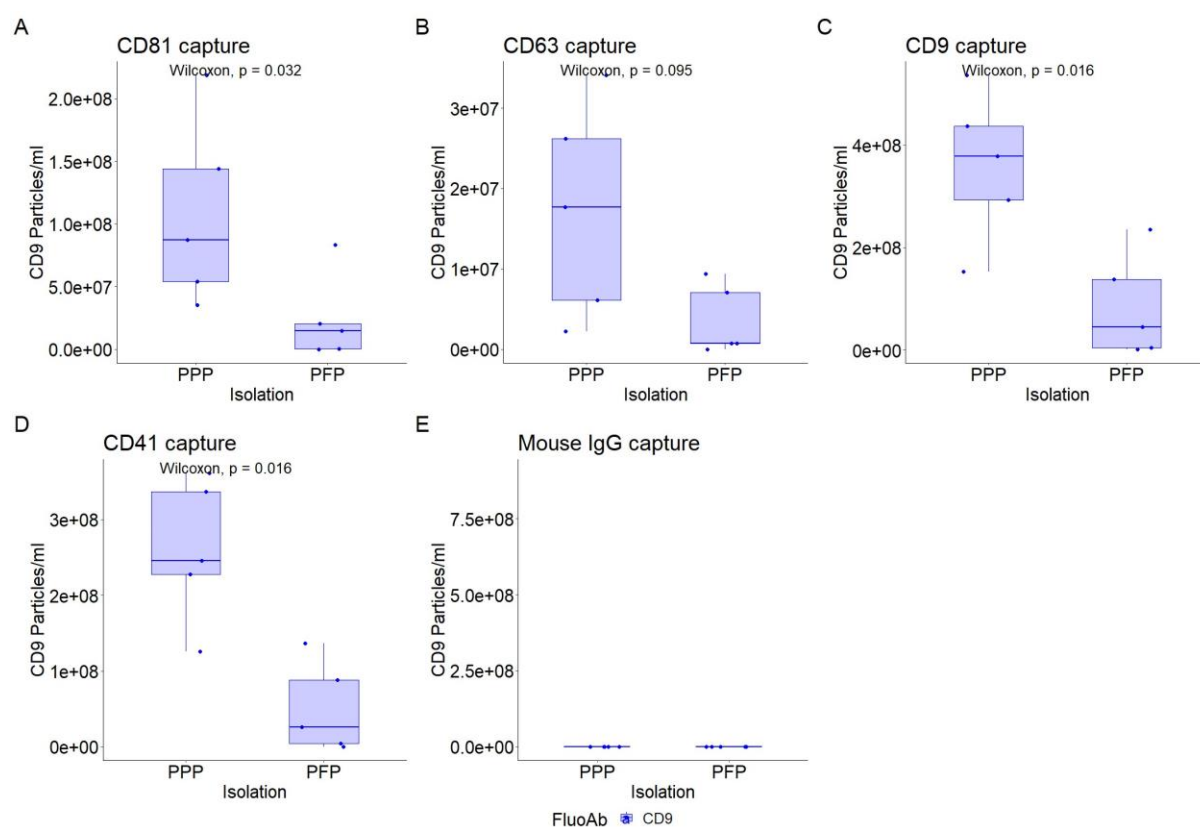


Figure 5.5. Detection of CD9 positive extracellular vesicles (EV) in platelet poor plasma (PPP) and platelet free plasma (PFP).

ExoView fluorescent EV count (anti-CD9-AF488) on PPP and PFP with results stratified by capture marker: a) CD81 ($p = 0.032$) b) CD63 c) CD9 ($p = 0.016$) d) CD41a ($p = 0.016$) e) Mouse IgG control, $n = 5$.

Having confirmed the PEV associated drop in EV count in PFP compared to PPP I next conducted a preliminary investigation and found that despite the significant reduction in platelet concentration in filtered PFP, there was no difference in EV concentrations between filtered and unfiltered PFP using capture against CD81, CD63, CD9 or CD41 (data not shown) and no difference in GPVI, CLEC2 or LAMP1 expression. As LAMP1 is thought to be a platelet EV specific marker, the hypothesis was that a large reduction of LAMP1 expressing EV would be observed in filtered PFP. CLEC2 may be slightly reduced following filtering as it is theoretically found on both PEV and MK EV. GPVI EV concentration would remain the same with and without filtering of PFP as GPVI is thought to be MK EV specific and not found on PEV. GPVI could therefore theoretically act as a control to ensure no loss of plasma EV as a result of filtering.

5.2.2 Characterisation of EV from activated platelets

Platelets were isolated from healthy volunteers and stimulated with 20 μ M ADP, 20 μ M TRAP or 10 μ M calcium ionomycin for 10 minutes at ambient room temperature. Platelet activation was measured using the classical alpha granule degranulation marker CD62p by flow cytometry. The remaining platelet preparations were then centrifuged to obtain platelet free supernatants for storage and measurement of PEV. Platelet activation levels are shown in figure 5.6. Unstimulated platelets gave a background of 1.4% CD62p expression with an MFI of 16 a.u.. ADP treatment increased CD62p expression with 26.8% ($p = 0.005$) activation and an MFI of 322 a.u. ($p = 0.0022$). TRAP treatment increased CD62p to 69.5% ($p = 0.005$) with an MFI of 1265 a.u. ($p = 0.0022$). Unsurprisingly, ionomycin gave the highest CD62p expression with 94.5% ($p = 0.0048$) activation and an MFI of 4174 a.u. ($p = 0.0022$).

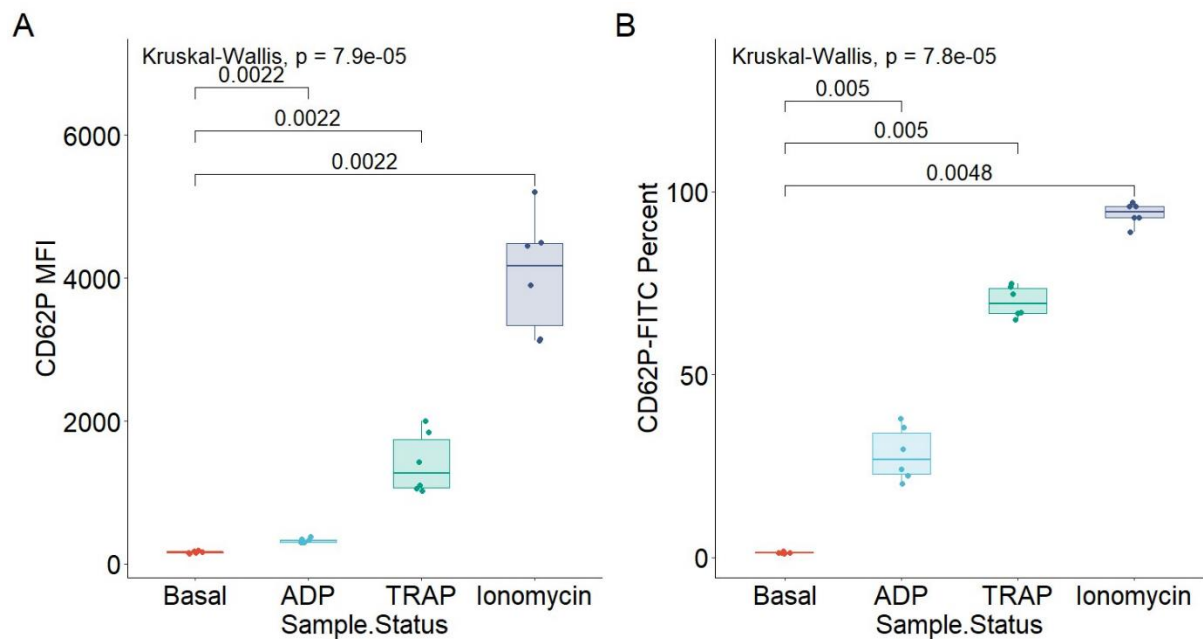


Figure 5.6. CD62P expression on purified platelets activated by various agonists.

CD62p activation marker levels were measured on basal unstimulated platelets and following stimulation with either 20 μ M ADP, 20 μ M TRAP (thrombin receptor activating peptide) or 10 μ M calcium ionomycin a) Anti-CD62P antibody (CLBThromb/6-FITC) median fluorescence intensity, global $p = 0.000079$, $n = 6$. b) Anti-CD62P antibody (CLBThromb/6-FITC) % expression on platelets, global $p = 0.000078$, $n = 6$.

Having determined that ADP, TRAP and ionomycin stimulation increased expression of the degranulation marker CD62P, the total number of EV were initially measured in the platelet supernatants by NTA.

As shown in Figure 5.7, ionomycin stimulation increased the total EV concentration compared to basal levels (6.8×10^8 particles/ml vs 4.5×10^9 particles/ml, $p = 0.0079$). However, ADP ($p = 0.42$) and TRAP ($p = 0.31$) did not increase total EV concentrations. Furthermore, no significant differences were observed in the size distributions of EV across any condition tested ($p = 0.12$). Based on these results basal and calcium ionomycin stimulated platelet supernatants were moved forward into ExoView testing with tetraspanin plasma chips.

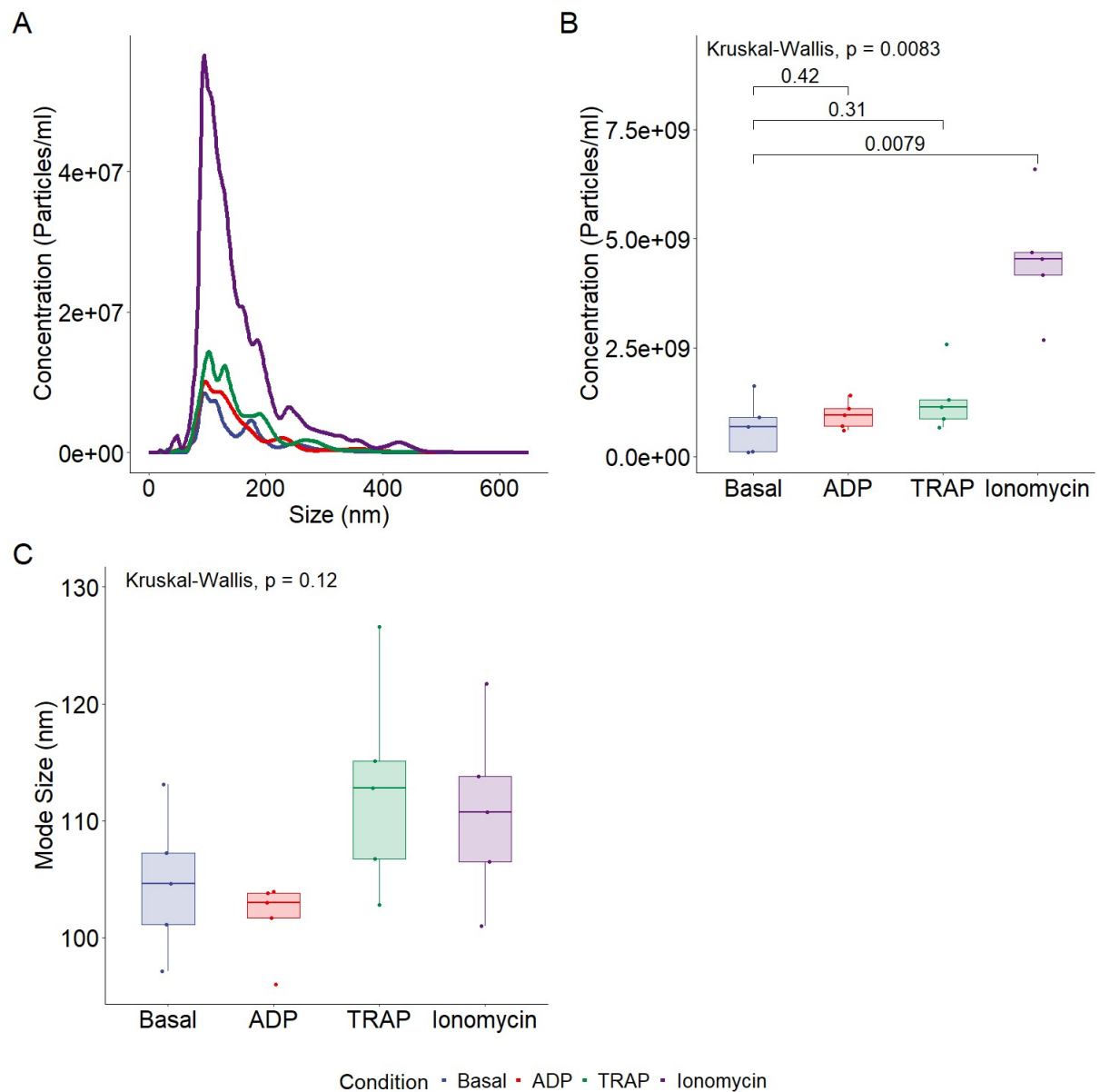


Figure 5.7. Measurement of the size and concentrations of PEV within stimulated purified platelet supernatants by nanoparticle tracking analysis (NTA).

EV concentration of platelet supernatants following stimulation with either 20 μM ADP, 20 μM TRAP (thrombin receptor activating peptide) or 10 μM calcium ionomycin. a) Size-concentration line graph (Median of $n = 5$) b) Concentration of EV (global $p = 0.0083$, $n = 5$) c) Mode size of particles ($n = 5$)

ExoView interferometric imaging EV concentrations within basal and ionomycin stimulated platelet supernatants are shown in Figure 5.8. CD81 capture showed no significant differences between basal and ionomycin stimulated platelets. There was a significant increase in EV captured from ionomycin stimulated platelet supernatant compared to basal captured by CD63 (4.1×10^7 vs 4.9×10^6 particles/ml, $p = 0.022$), CD9 (8.1×10^7 vs 9.2×10^6 particles/ml, $p = 0.022$) and CD41 (9.2×10^7 vs 2.7×10^7 particles/ml, $p = 0.022$). Interestingly, there are orders of magnitude difference between NTA provided concentrations and ExoView concentrations. NTA concentrations are in the $\times 10^9$ range, whereas, ExoView concentrations range between $\times 10^6$ - $\times 10^8$ range. This reflects the difference between bulk measurement and targeted capture.

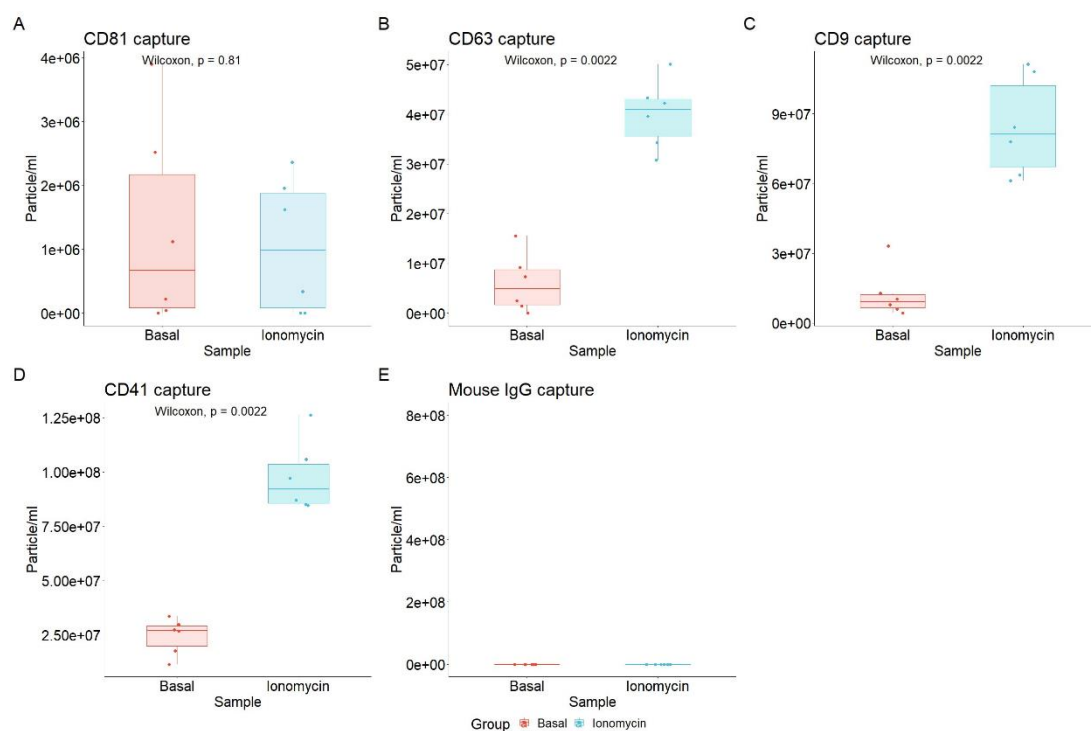


Figure 5.8. Extracellular vesicle (EV) concentrations in platelet supernatants by ExoView interferometric analysis.

a) ExoView EV interferometric analysis of EV concentration in basal and 10 μ M calcium ionomycin stimulated platelet supernatants with results stratified by capture marker: a) CD81 b) CD63 ($p = 0.0022$) c) CD9 ($p = 0.0022$) d) CD41a ($p = 0.0022$) and e) Mouse IgG control, $n = 6$.

With secondary fluorescent labelling of basal and ionomycin stimulated purified platelet supernatants, GPVI, CLEC2 and LAMP1 expression were also measured by ExoView. Figure 5.9 shows no significantly different levels of GPVI expression when measured by secondary labelling with CD81 captured EV. There were increased concentrations of GPVI expressing EV with CD63 capture (2.1×10^5 particles/ml vs 1.6×10^6 particles/ml, $p = 0.013$), CD9 capture (4.4×10^5 particles/ml vs 3.5×10^6 particles/ml, $p = 0.02$) and CD41 capture (3.0×10^6 particles/ml vs 1.2×10^7 particles/ml, $p = 0.041$) with ionomycin stimulation. Figure 5.10 shows no significantly different CLEC2 expression when measured by secondary labelling with CD81 captured EV. There were increased concentrations of CLEC2 expressing EV in ionomycin stimulated platelet supernatants captured with CD63 (0.0 particles/ml vs 3.0×10^6 particles/ml, $p = 0.0043$), CD9 (7.4×10^5 particles/ml vs 1.1×10^7 particles/ml, $p = 0.0081$) and CD41a (3.0×10^6 particles/ml vs 3.0×10^7 particles/ml, $p = 0.0043$). No significant differences were found for LAMP1 expression between basal and calcium ionomycin stimulation (Figure 5.11).

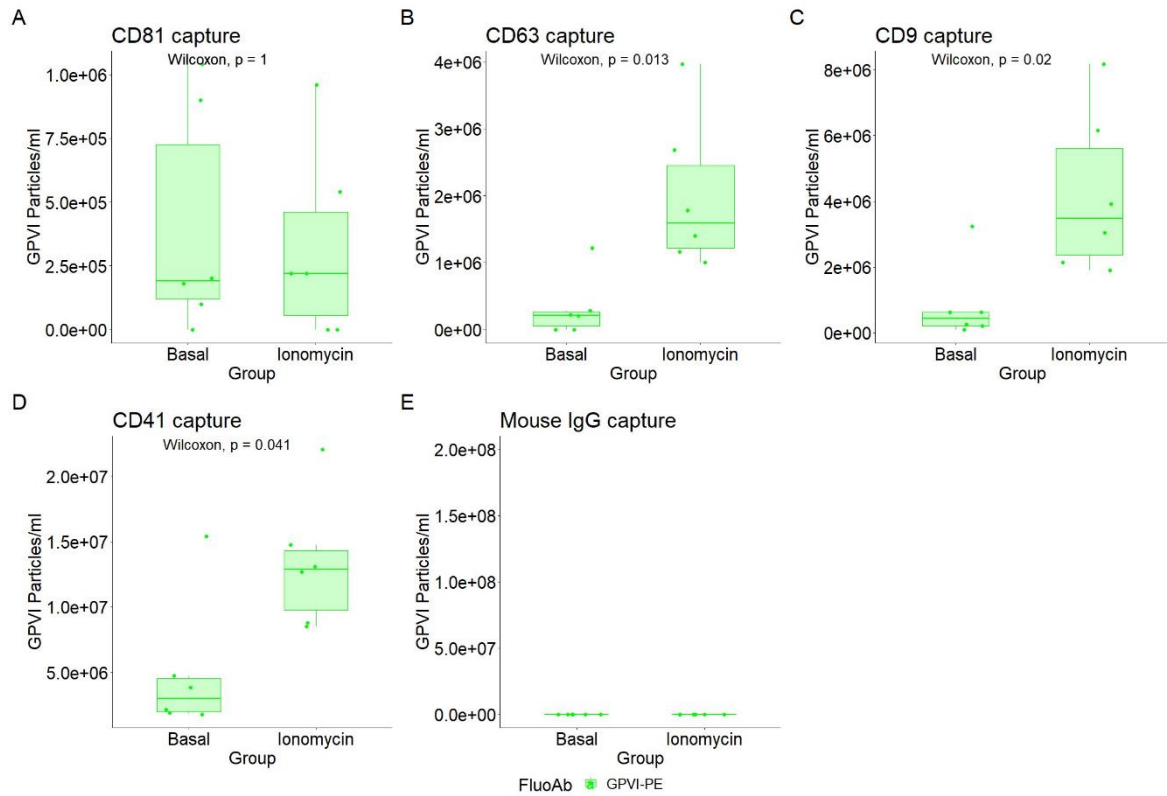


Figure 5.9. Washed platelet supernatant GPVI positive extracellular vesicle (EV) concentrations.

ExoView fluorescent EV concentration (anti-GPVI-PE) in basal and 10 μ M calcium ionomycin stimulated platelet supernatants. a) CD81 b) CD63 ($p = 0.013$) c) CD9 ($p = 0.02$) d) CD41a ($p = 0.041$) e) Mouse IgG control, $n = 5$.

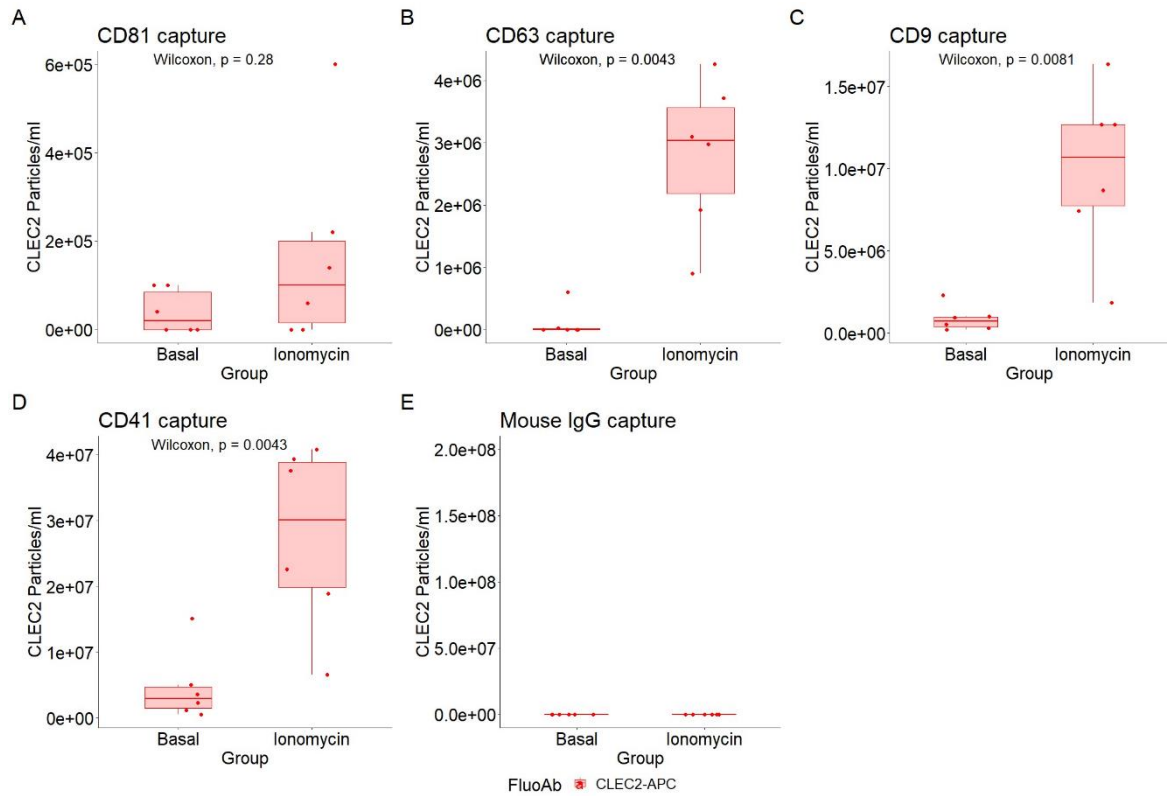


Figure 5.10. Washed platelet supernatant CLEC2 positive extracellular vesicle (EV) concentrations.

ExoView fluorescent EV concentration (anti-CLEC2-APC) in basal and 10 μ M calcium ionomycin stimulated platelet supernatants. a) CD81 b) CD63 ($p = 0.0043$) c) CD9 ($p = 0.0081$) d) CD41a ($p = 0.0043$) e) Mouse IgG control, $n = 5$.

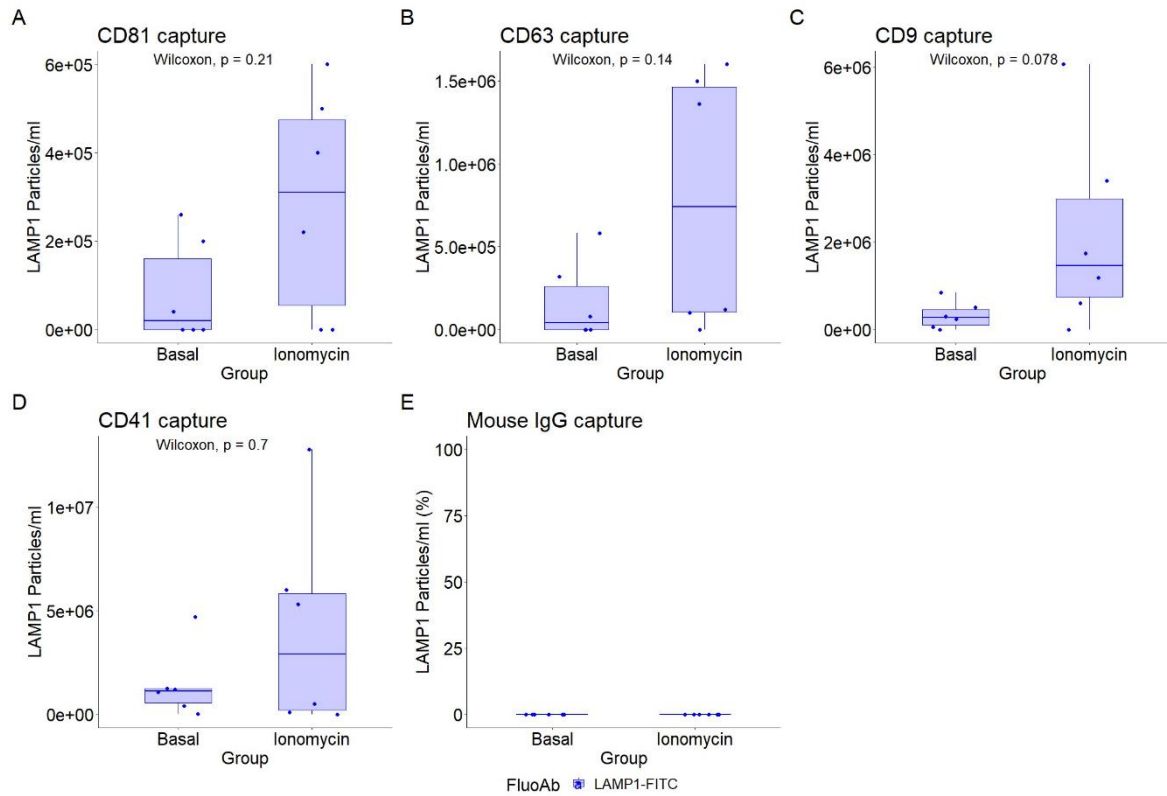


Figure 5.11. Washed platelet supernatant LAMP1 positive extracellular vesicle (EV) concentrations.

ExoView fluorescent EV concentration (anti-LAMP1-FITC) in basal and 10 μ M calcium ionomycin stimulated platelet supernatants. a) CD81 b) CD63 c) CD9 d) CD41a e) Mouse IgG control, n = 5.

5.2.3 Comparison of PFP, platelet and MK EV

MK cell culture supernatants containing EV were kindly gifted by Professor Cedric Ghevaert from the University of Cambridge. MK EV were measured on ExoView in comparison with PFP and fluorescently labelled with GPVI, CLEC2 and LAMP1. Most ExoView data is normally expressed as particles/ml, but in the case of comparing PFP with activated platelet supernatants and MK EV the differing origins (and cell concentrations) of these biofluids precludes this form of comparison, because the number of MK derived EV in cell culture cannot be directly compared with PFP or with activated platelet supernatants. For this reason, each fluorescent marker was expressed as a percentage of the total population of fluorescently counted EV. Percentage expression thus facilitates a direct comparison of the relative composition of EV from differing biofluids. The composition of EV from PFP, basal or ionomycin stimulated platelets and MK EV were not significantly different either with CD63, CD9 or CD41 capture for GPVI expression. For CD81 capture, PFP had a higher percentage of GPVI expression (87.1%) compared to ionomycin stimulated platelet supernatants (31.5%, $p = 0.013$) (Figure 5.12). Ionomycin stimulated platelet supernatant (31.5%) GPVI expression is lower than basal platelet supernatants (73.2%, $p = 0.044$) and MK EV (70.4%, $p = 0.022$) (Figure 5.12). The composition of CLEC2 EV from PFP, basal or ionomycin stimulated platelets and MK EV were not significantly different with CD81, CD9 or CD41 capture (Figure 5.13). For CD63 capture CLEC2 expression was higher in ionomycin stimulated platelet supernatant (55.5%) than PFP (15.4%, $p = 0.0056$) and basal platelet supernatants (0%, $p = 0.013$)

(Figure 5.13). MK EV (37.9%) also had higher CLEC2 expression than basal platelet supernatants (0%, $p = 0.034$). The composition of EV from PFP, basal or ionomycin stimulated platelets and MK EV were not significantly different with CD81, CD9 or CD41 capture for LAMP1 expression (Figure 5.14). For CD63 capture, LAMP1 expression on MK EV (0%) was lower than PFP (36.4%, $p = 0.0083$), and ionomycin stimulated platelets (10.6%, $p = 0.04$) (Figure 5.14).

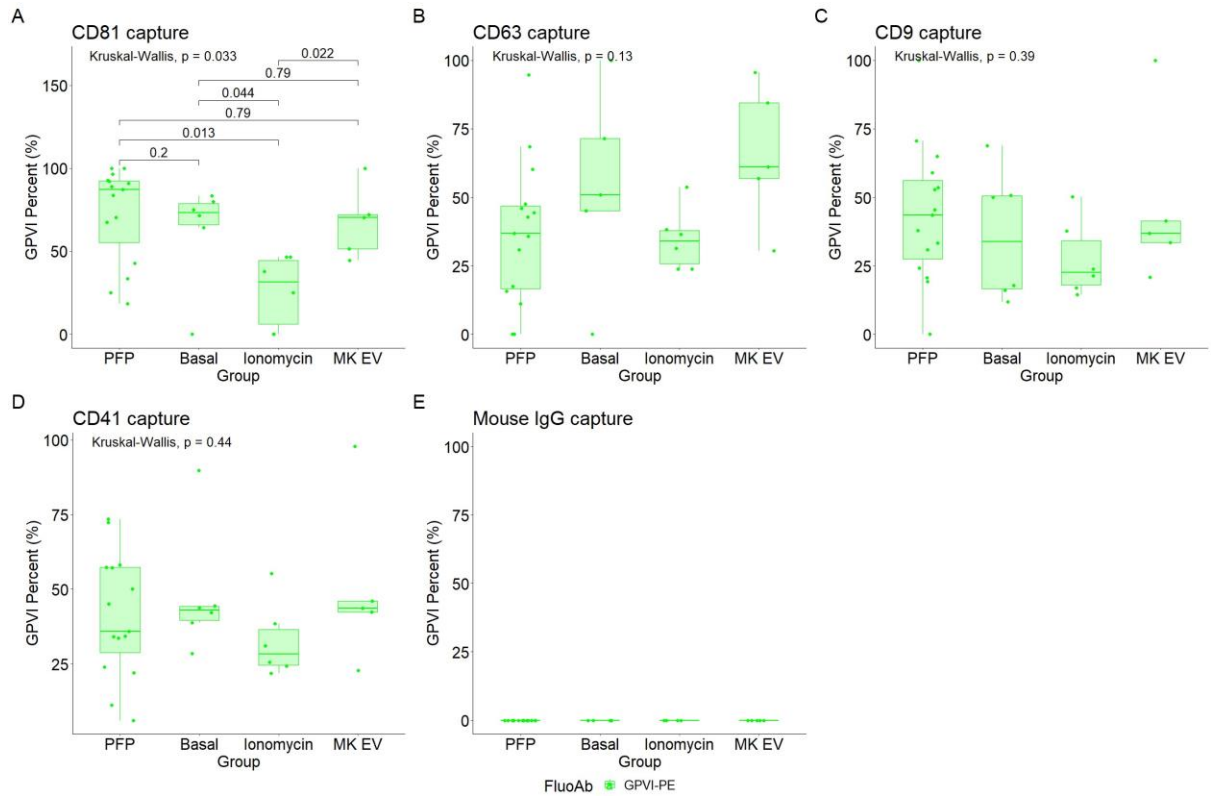


Figure 5.12. Percentage of GPVI positive extracellular vesicles (EV) in platelet free plasma (PFP), basal/calcium ionomycin stimulated washed platelet supernatants and megakaryocyte (MK) EV.

ExoView fluorescent EV percentage (anti-GPVI-PE) with results stratified by capture marker: a) CD81 (global $p = 0.033$) b) CD63 c) CD9 d) CD41a e) Mouse IgG control (E), $n = 15$ for PFP, $n = 6$ for basal, $n = 6$ for calcium ionomycin $n = 5$ for MK EV.

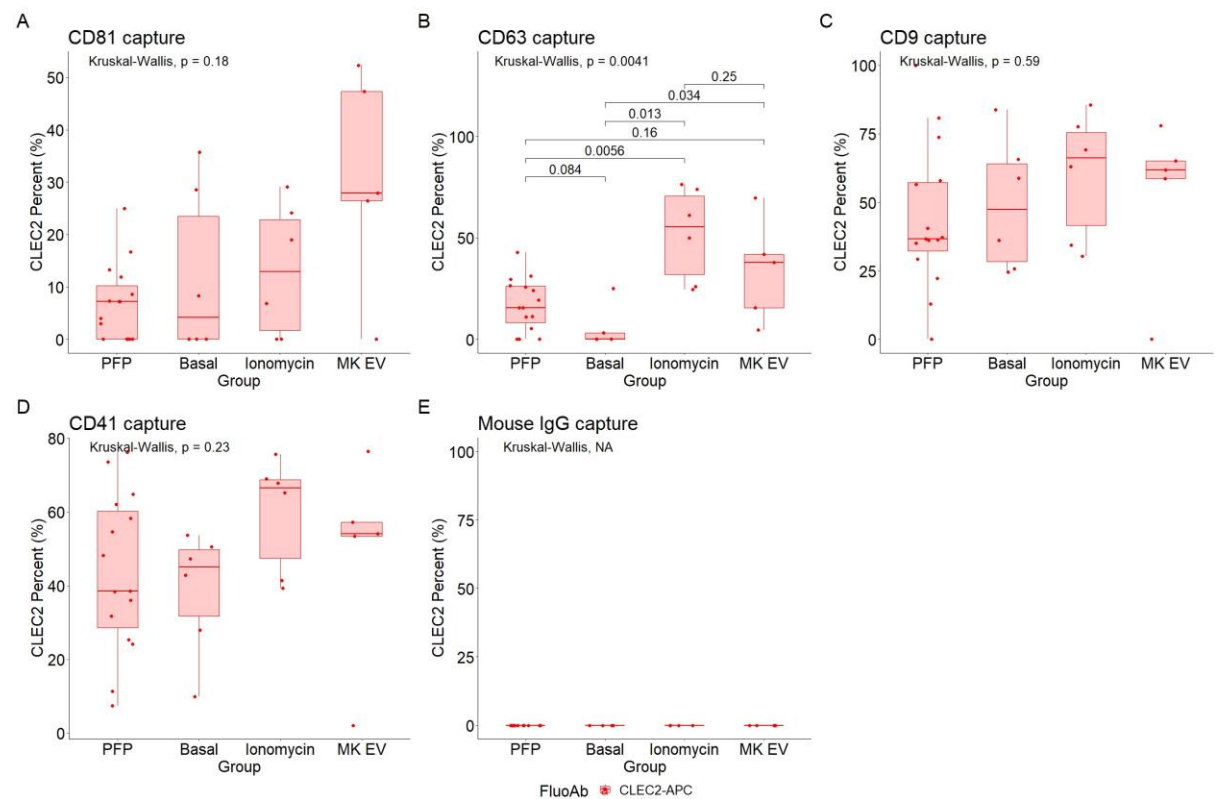


Figure 5.13. Percentage of CLEC2 positive extracellular vesicles (EV) in platelet free plasma (PFP), basal/calcium ionomycin stimulated washed platelet supernatants and megakaryocyte (MK) EV.

ExoView fluorescent EV percentage (anti-CLEC2-APC) with results stratified by capture marker: a) CD81 b) CD63 (global $p = 0.0041$) c) CD9 d) CD41a e) Mouse IgG control (E), $n = 15$ for PFP, $n = 6$ for basal, $n = 6$ for calcium ionomycin $n = 5$ for MK EV.

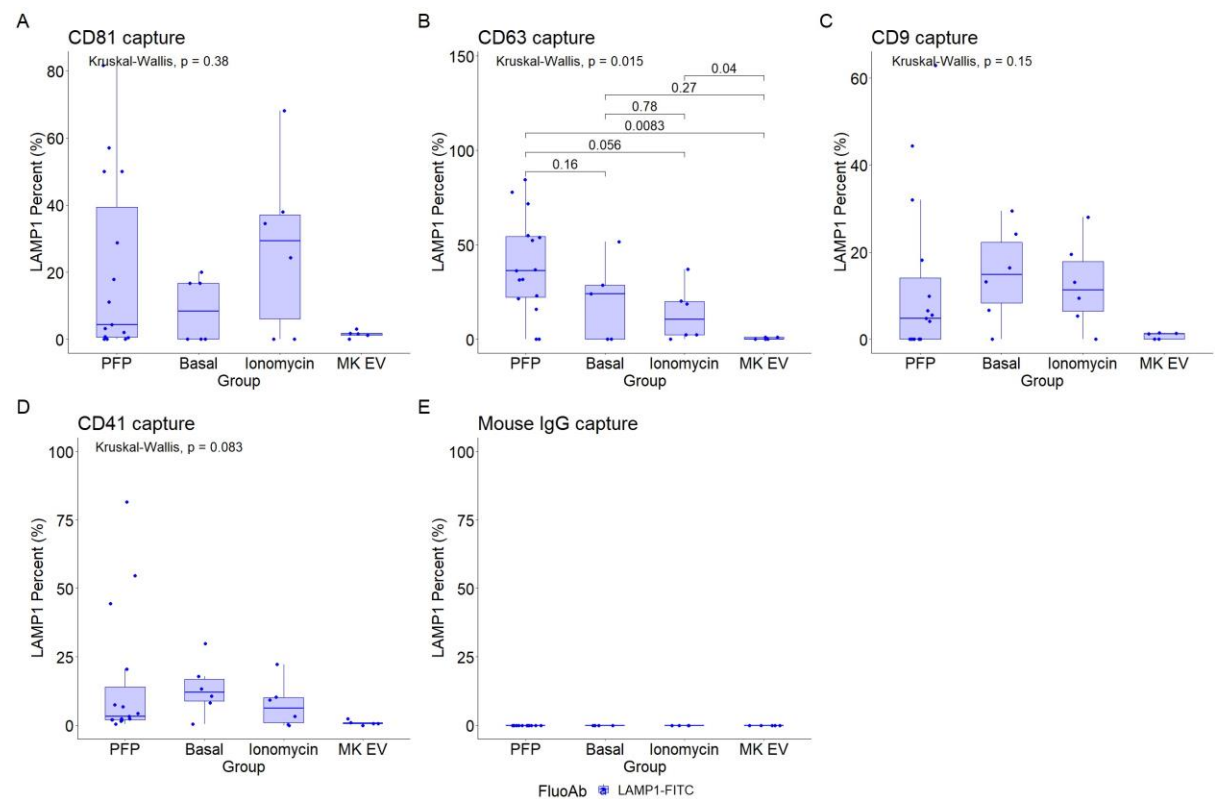


Figure 5.14. Percentage of LAMP1 positive extracellular vesicles (EV) in platelet free plasma (PFP), basal/calcium ionomycin stimulated washed platelet supernatants and megakaryocyte (MK) EV.

ExoView fluorescent EV percentage (anti-LAMP1-APC) with results stratified by capture marker: a) CD81 b) CD63 (global $p = 0.015$) c) CD9 d) CD41a e) Mouse IgG control (E), $n = 15$ for PFP, $n = 6$ for basal, $n = 6$ for calcium ionomycin $n = 5$ for MK EV.

5.4 Discussion

In this chapter, I confirm what is already well-established in the literature, that PPP prepared by a single centrifugation step contains residual platelets and their presence leads to an artefactual increase in PEV that are detectable using ExoView. I also confirm the less well known and published but unsurprising finding that PFP still contains remnant platelets. Even though there is a significant depreciation in platelet concentration following 0.8 μm filtration of PFP, the ExoView data suggests there is no difference in EV concentration between PFP and filtered PFP. This finding has multiple interpretations with one being that remnant platelets in PFP are not sufficiently concentrated enough to contribute to freeze-thaw mediated PEV increases, or alternatively that ExoView is possibly not sensitive to measure the difference. These results are in line with Nieuwland's group who found no decrease in PEV (measured by flow cytometry) despite a reduction in platelet count post-filtering [237, 238].

I used flow cytometry to assess the activation status of platelets after *ex-vivo* agonist treatment. ADP, TRAP and calcium ionomycin increased CD62P expression in a stepwise manner thus verifying that platelets are activated. However, NTA showed that only ionomycin increased EV release. As ADP is a relatively weak agonist this is not expected to increase PEV concentrations. TRAP results in good degranulation and would be expected to release exosomal PEV. Although ionomycin is a strong agonist the PEV generated in this way may not be normal as it is non-physiological. Nonetheless, basal and ionomycin stimulated platelet supernatants were measured on ExoView. Ionomycin stimulated platelet

supernatants led to increased EV concentrations both with interferometric imaging and with secondary fluorescent labelling. Surprisingly, GPVI increased on CD63, CD9 and CD41 capture with ionomycin stimulation. GPVI is thought to be absent on PEV due to shedding. The relevance of GPVI expression on PEV derived from ionomycin stimulated platelets is low but is made interesting by the observation that unstimulated platelets are also releasing detectable concentrations of GPVI expressing PEV. The Italiano group found no evidence of PEV release in the absence of stimulation, whereas my data suggest there is. It is possible that there was EV contamination as part of the platelet purification process, however I think this is unlikely as the highest centrifugal force used to isolate the platelets was 1000 g for 10 minutes, where minimal EV would be pelleted. This leaves two explanations, GPVI EV are pelleted with platelets or more likely platelets can constitutively release PEV without stimulus. The literature claim that GPVI is an MK specific EV marker and absent from platelet EV may therefore not be correct. CD62P which was initially thought to be PEV specific and absent on MK EV is now recognised to be present on both MK EV and PEV. I think the data I present suggests GPVI is similar to CD62P in that it cannot be used to differentiate PEV and MK EV. That said, the percentage expression of LAMP1 is minimal on MK EV and may negatively identify MK EV. However, it is not a true discriminatory marker as it does not faithfully identify all PEV. This data points to no simple answer to the primary origins of circulating EV and therefore is likely to be a mixture of both PEV and MK EV. I think that future work in this area would benefit from taking a different approach, namely proteomic analysis of EV from plasma, MK EV and platelet supernatant EV. In theory this could provide a large list of differentially expressed

proteins which could then be validated through ELISA, western or indeed ExoView. The most promising markers could also be viewed in principal component analysis plots which may hint at whether plasma EV share more or less similarity with MK EV or PEV. If this research were fruitful it could be followed up in thrombocytopenic patients and CVD patients, with my hypothesis being that thrombocytopenic patients would have less PEV and CVD patients would have more. A key aspect of this type of research would be a greater focus on physiologically relevant agonists of platelets.

Chapter 6. Chapter 6. General Discussion

6.1 Discussion

EV measurement is challenging. Historically, EV often require isolation prior to measurement thus introducing bias in the studied populations [243]. Furthermore, the small size of EV limits many conventional methods of detection, with conventional flow cytometry only capable of resolving approximately 1-10% of total EV [374]. Even with instruments (e.g. NTA) that can resolve small particles, they often fail to distinguish EV from other similarly sized particles such as lipoproteins [243]. At the outset of my thesis the ExoView R100 was a new instrument that was potentially promising in its ability to specifically capture and measure EV in complex biofluids without the need for isolation steps and without misattributing other small particles as EV [216].

My thesis aims initially were to test whether ExoView can measure plasma EV with a specific focus on EV that may act as biomarkers for inflammation, thrombosis and bleeding risk. This would have involved use of PFP samples from the longitudinal observational CASCADE cohort. This trial was powered to recruit 1,065 participants to detect a 15% increase in risk of spontaneous bleeding associated with biomarker measurements, including EV. A sample size of 1,184 patients was therefore chosen for the treatment group to allow for 10% loss to follow-up. For the EV aspect of this research, I had planned to initially compare 44 patients treated with antiplatelet/anticoagulant therapy and 44 age and sex matched controls that were not treated with antiplatelet/anticoagulant therapy. Unfortunately, COVID-19 pandemic measures were introduced shortly into the start of trial recruitment leading to low numbers of participants and minimal follow-up sampling meaning the primary hypothesis could not be tested. Despite this, COVID mitigations were put in place and

my thesis aims were revised to focus on measurement of procoagulant PS EV in plasma, characterisation of PEP as a potential novel EV based resuscitation fluid, measurement of TF positive EV in plasma and to address whether PEV and MK EV can be distinguished from each other.

To start with, I first had to test whether ExoView was capable of measuring EV in plasma without the need for isolation. It would be ideal to compare the ExoView R100 to a gold standard, but alas there is no such instrument available yet for the EV biologist. Instead, I started by generating a PEV rich material (PEP) with high procoagulant activity mediated by PS. This was measured by ROTEM and CAT and shown to have efficacy in restoring clotting time in an *in vitro* model of dilutional coagulopathy. PEP therefore was a complex plasma-based biofluid rich in PEV with specific procoagulant function. Having confirmed the functional PS-mediated procoagulant activity of PEP from PEV I then used this sample to validate that ExoView is also capable of detecting this. The work up of an ExoView compatible method to measure PS on captured EV using annexin-V labelling was difficult and involved a great deal of troubleshooting, which in retrospect now seems somewhat avoidable. Physiological calcium concentrations are required for annexin-V to bind PS, the realisation that adding calcium to diluted plasma enriched with PS-expressing EV would lead to thrombin generation and fibrin formation on the chip now seems obvious. GPRP-amide could have been used from the outset of these experiments or alternatively utilising the calcium independent fluorescently labelled probe lactadherin may have been a good approach. However, the fibrin formation is independently interesting as it was notably increased on CD41 capture compared to CD81 capture.

I interpret this as possible EV binding to fibrin via integrin $\alpha\text{IIb}\beta 3$ and then binding specifically via the EV to specific capture spots (e.g. CD41). Additionally, in light of Chapter 5 showing GPVI expression on activated PEV, it is possible that EV associated GPVI may have also contributed to EV-fibrin binding due to the known interactions of GPVI with fibrin in clots [375]. This possibly provides more supporting evidence to EV involvement in the procoagulant function and formation of fibrin clots [376]. However, this does have to be weighed carefully against the ROTEM dilutional coagulopathy model data. PEP treatment of diluted hypothermic blood does not increase the MCF, therefore although PEV are incorporated into clots they do not increase clot quality independently. However, the high surface area:volume of ratio of PEV and their expression of markers such as GPVI and integrin $\alpha\text{IIb}\beta 3$ may allow for their inclusion in clots where they can rapidly increase thrombin generation and reduce time taken to clot [377]. This may have been relevant had I measured the CASCADE samples, PEV increases have been associated CVD [378-382], however, the balance of markers on PEV in these patients have largely not been assessed and may be critical in understanding differential patient outcomes in response to antithrombotic therapies [383, 384].

I also performed an investigation into whether commercially available platelet lysates exhibited the same characteristics as PEP derived from PRP. Commercial HPL exhibited reduced clotting despite their high thrombin generation capacity, a phenomenon that was reversible in a dose-dependent manner through fibrinogen supplementation, this may be unsurprising as HPL are designed for use in cell culture where clot formation is undesirable [385, 386]. Interestingly, using the PEP procedure

on NHSBT platelet units did not show the same results as commercially available HPL. NHSBT expired platelet unit derived PEP at day 12 of platelet ageing showed poor functionality with aberrant CAT traces and poor CT values (ROTEM). Whereas 8-day old platelet units exhibited properties similar to that of PRP derived PEP with similar clotting and thrombin generation capacity as measured by ROTEM and CAT, respectively. This suggests that the PEP procedure could be used to extend the lifecycle of donated platelet units but only if utilised at day 8 or before.

To get PEP to clinical use, many more hurdles will have to be overcome. To start with a time course following platelet ageing, PEP generation and functional activity will be necessary to determine the optimal platelet age. A full proteomic analysis, measurement of serotonin, plasminogen activator 1 (PAI-1) and polyphosphate would be required [345]. PEV interaction with leukocytes, the endothelium and platelets will also be important parameters [164]. An additional challenge with EV based therapeutics is establishing dosage and ensuring a standardised final product [346]. PEP is proof of principle that PEV may have therapeutic uses, which adds to the growing literature that supports the use of PEV clinically [147, 162, 168, 169, 387]. A key difference between PEP and other PEV-based treatments is the inclusion of plasma. Balanced salt solution suspensions that hold therapeutic PEV may aid in reducing clotting time and dilutional coagulopathy but saline has been repeatedly shown to worsen metabolic acidosis compared to plasma [388, 389]. PEP may also improve metabolic acidosis through the inclusion of plasma.

A final PEP preparation derived from platelet units could be investigated through *ex vivo* treatment of blood from trauma patients with coagulopathy, followed by

investigation in an *in vivo* small animal model of trauma or haemorrhage. If PEP performs well in animal models of haemorrhage it may also be worth considering lyophilisation for easy storage. A lyophilised version of PEP that is stable at ambient temperature could provide enormous utility for resuscitation in remote or austere environments such as for military purposes [390, 391]. Freeze-drying PEP is highly plausible as lyophilised plasmas are already in use and have been shown to be as efficacious, if not more efficacious than FFP in clinical trials on trauma [392, 393]. Interestingly, the idea of using platelet lysates therapeutically is growing with one paper using ROTEM and confirming the findings of PEP [394].

I was lucky enough to be involved in characterising procoagulant EV from a rare Scott syndrome patient. This utilised similar methods to the PEP work for detecting PS-positive EV and highlights the importance of thrombin generation mediated by platelets and potentially EV in the clinical setting. The Scott syndrome family also allowed me to test whether circulating and/or *ex vivo* stimulated PEV expressed PS in individuals that were homozygous or heterozygous for a novel ANO-6 mutation. PFP from the Scott syndrome homozygous patient showed less capacity to generate thrombin compared to their heterozygote mother and healthy controls. This was also confirmed antigenically with the ExoView also showing a reduction in the concentration of EV captured on CD41 that express PS in PFP. Platelets normally respond through externalisation of PS on their surface and on their released EV in response to appropriate stimuli that cause sustained calcium activation and cytoskeletal remodelling [149, 352]. Flow cytometry however demonstrated minimal externalisation of PS on the homozygous patient platelets following stimulation with CRP or ionomycin

compared to the heterozygous mother. Interestingly, isolating purified platelets from the patient and stimulating them to generate PEV showed no increase in PS expression. This work follows on from the 1989 documentation of reduced PEV from Scott syndrome patients [353]. An important unresolved question in coagulation and thrombin generation is the relative importance or input of PEV vs platelets to thrombin generation via PS exposure. One estimate suggests that PEV released from activated platelets have 50-100 fold higher procoagulant activity compared to activated platelets [395]. However, it has been suggested that surface area and circulating PEV calculations can be used to calculate the relative importance of PEV in procoagulant activity [144], however without linking to thrombin generation or functional outputs, this may be misrepresenting PEV contribution as they are likely only released upon platelet activation [186]. This leads to potentially erroneous conclusions, as Chapter 5 shows, PEV and MK EV share many similarities and are difficult to distinguish from each other.

Taken together, the PEP and Scott syndrome data suggest that PS-expressing PEV may hold an important role in physiological clot formation in health and disease acting to rapidly aid platelets in forming stable clots. It is feasible that in the CASCADE cohort PS-expressing PEV could have been a critical biomarker of bleeding or thrombosis risk. Interestingly, a recent publication found that ticagrelor treatment of MI patients was associated with reduced PEV concentrations compared to clopidogrel [396]. Additionally, over the 6 months of follow up PS-expressing EV concentration increased with both ticagrelor and clopidogrel but to a lesser extent with ticagrelor. That study was not powered to detect clinical outcome differences and it would be beneficial for future studies to determine if PEV concentration was associated with adverse clinical

outcomes. The fact that PS-expressing PEV concentrations increased on both treatments is also worthy of follow-up, PEV may represent a therapeutic target that could reduce poor clinical outcomes for CVD patients. However, no healthy age-matched controls were used in the study by Gasecka *et al* and this should be an important inclusion for follow-up work [396].

It is also worth considering that pharmaceutical agents outside of the anti-platelet realm may have effects on PEV generation. Lipid raft formation has been shown to be critical for release of PS-expressing PEV, but is not required for externalisation of PS on activated platelets [397]. Inhibiting lipid raft formation, for instance through cholesterol depletion, reduces PS-expressing PEV biogenesis but still allows for externalisation of PS on platelets [397]. Deciphering the relative importance of PS externalisation on platelets and PEV to procoagulant activity, combined with lipid membrane altering pharmaceutical agents, potentially opens the door to modulating the platelet:PEV PS ratio which could aid in treatment of CVD patients. Furthermore, lipid rafts are also crucial in biogenesis of TF-expressing EV from monocytes, so lipid membrane modulation may be a target therapy that can affect multiple aspects of procoagulant EV biogenesis [398]. β -Cyclodextrin is a candidate therapeutic agent with cholesterol depleting properties that has already been used in mouse models for prevention of atherosclerosis but has been associated with side effects in the form of both nephrotoxic [399] and auditory nerve damage [400]. However, methyl- β -cyclodextrin is likely to be more efficacious as it has more specificity for plasma membrane lipid depletion [401].

Having characterised PEP and a Scott syndrome patient with a variety of methods and a focus on ExoView, a clear next step was to look at another key coagulation associated EV marker: TF. TF research alone has often been controversial as it is a trace protein, but combining TF and EV research together is even more challenging [192]. For that reason, great care was taken in designing experiments that followed the roadmap of TF EV measurement suggested by Gardiner *et al* and Nieuwland *et al* [214, 215]. The key problems in antigenic TF EV measurement are a historic lack of specificity with certain commercial and non-commercial sources of anti-TF antibodies, incongruent results between laboratories and the physiologically low concentrations of TF [192, 325]. For this reason, TF activity measurements are the preferred method of choice [222], but these are labour intensive and may be missing the potential importance of the measurement of encrypted TF with latent activity [200, 402]. Anti-TF antibodies have been broadly divided into 3 groups. Theoretically, only a combination of monoclonal antibodies that bind at distinct epitopes should be used for TF detection in the form of sandwich ELISAs [325, 359]. I determined that combining IID8-AF647 with an anti-TF antibody 5G9 capture system was a valid approach that demonstrated not only detection of TF on EV derived from the cancer cell line BxPC-3 but also on EV derived from LPS stimulated blood-derived monocytes. Although, clinical samples could not be distinguished using this assay I show proof of principle that ExoView has the potential to do this and show for the first time that the ExoFlex technology can aid in capturing rare EV populations by using customized antibodies. I also provide power calculations that aid in determining how many samples are required to detect differences in future similar studies.

Other antigenic approaches for TF detection have been used in recent years, including the MACSplex exosome kit (Miltenyi Biotec, Bergisch Gladbach, Germany) kits [403]. MACSplex exosome kit is a bead-based immunocapture system designed to capture EV on beads which can then be labelled with fluorescent antibodies against tetraspanins. The relatively large size of the beads compared to EV allows for conventional flow cytometry to assess EV concentration. Balbi *et al* used this method and found a significant increase in TF-expressing EV concentration in the serum of COVID patients [403], whereas Archibald *et al* later determined the signal for TF provided by MACSplex to be non-specific [264]. These results add to the confusion surrounding TF EV measurement. Furthermore, the MACSplex kit uses a clone of HTF-1 which is a highly validated TF antibody, Archibald *et al* show that the Mackman TF activity assay, which uses HTF-1, does not correlate with MACSplex TF data. However, Archibald *et al* uses only a small number of participants to make this conclusion [264]. Despite the ongoing problems with TF EV detection there remains great interest in TF EV detection for the stratification of ovarian, pancreatic cancer and glioblastoma patients at risk of venous thromboembolism (VTE) [71, 73, 207, 224, 404-406]. Interestingly, despite the current high reliance on TF activity assays, there is growing evidence that PS can regulate the structure and function of FVIIa, potentially determining whether FVIIa interacts with the alternately spliced variant form of TF [199, 205] with low proteolytic activity or the active version of TF [201]. This may be an *in vitro* artifact, or alternatively this could influence encryption status of TF and may have currently unmeasured effects on TF activity assays. With hindsight it might have been interesting to measure PS and TF together using the ExoView. Given the difficulty in standardising TF EV measurements and the controversy that surrounds

TF, perhaps alternate markers that are more easily detectable may be a more fruitful approach to developing a VTE stratification system. Glioblastoma derived EV may also be positive for both TF and podoplanin and thus cause VTE through both coagulation and platelet activation. ExoView measurement would be interesting to verify TF and podoplanin dual positivity in glioblastoma patients with VTE [406].

My final results chapter then highlights the continuing difficulty in specifically phenotyping circulating EV of either platelet and/or MK origin using the ExoView platform. This chapter was the most challenging aspect of my thesis, the common lineage of platelets and MK limits the unique markers their EV possess, which are then further compounded by historic platelet contamination of plasma which artificially increases the PEV count [237, 238]. However, there were promising specific markers in the form of GPVI as specific to MK EV and LAMP1 as specific to PEV. The leading theory is that MK EV are the dominant circulating EV population in health and that they are released constitutively, whereas PEV are released largely only in states of inflammation and disease upon platelet activation where they contribute to CVD [186]. If the scientific community had efficient ways to resolve PEV and MK populations they may be able to apply the ratio of MK EV:PEV as a marker of CVD and to inform pharmaceutical treatments. Despite the previous identification of promising specific markers from the literature, I show that the ExoView technology cannot be used to distinguish PEV and MK EV under the conditions I tested. I aimed to generate specific EV “signatures” of platelet and MK origin but found that it was not straightforward to conclusively define exclusive and specific signatures for either PEV or MK EV. The data would suggest that there are no dominant PEV or MK EV populations and instead

suggest that this circulating EV pool is a heterogeneous mix of PEV and MK EV with high similarity and overlap in expressed markers. Alternatively, the small specific signal of LAMP1 seen only with stimulated PEV may be useful and relevant. Potentially, the GPVI increase observed in ionomycin stimulated PEV is an artefact of non-physiological stimulation. A different future approach to this problem could involve the use of functional measures such as CAT and include annexin-V labelling on ExoView which may further aid in identifying PEV, however PS expression does not occur on all PEV and would still leave a population of indistinguishable PEV/MK EV [407]. Next steps for this work would be to assess whether GPVI PEV concentration increases with more physiologically relevant agonists, including collagen, thrombin and combinations of both. If GPVI expression is more limited to MK EV than my data suggests then it may be possible to generate a multidimensional model that acts as a ratio of activated PEV:MK EV. This would also have to be confirmed in a cohort similar to CASCADE. An approach like this would benefit from measurement of full length filamin which is theoretically MK EV specific, and cleaved filamin which is theoretically PEV specific [187]. Greater resolution may be possible following proteomic analysis of PEV and MK EV, this would aid in identifying not necessarily unique markers of each population but differentially expressed markers. Principal component analysis of proteomic data from PEV and MK EV will help in establishing the actual overlap of PEV and MK EV populations. If they can be accurately distinguished, a new larger panel can be made. Following verification on PEV and MK EV, data output could be applied to machine learning models which would be trained to identify PEV and MK EV. This model could then be applied to plasma from healthy controls and patients with CVD. For this approach to work the most important aspect will be the quality of

the data used, it will be important to use more platelet agonists at more concentrations to develop a fuller view of PEV markers. It is also possible that EV biogenesis of purified platelets (in balanced salt solutions) is different to platelets suspended in plasma, it would be interesting to use UC on PFP to generate EV-depleted PFP and use the EV-depleted PFP to resuspend isolated platelets and assess EV signatures in response to stimulation.

Although I could not successfully resolve PEV and MK EV, measurement of specific EV of unique cellular origin may have application in other areas including in the post-doctoral research I'm currently engaged in. For example, synovial fluid is complex and has EV that likely originate from synovial fibroblasts, chondrocytes, osteoblasts and other cells of the joint. In rheumatoid arthritis, it has been shown that PEV play an important role in joint inflammation [143]. In the case of osteoarthritis this has not yet been shown. Additionally, EV may also contribute to cartilage degradation, so as well as phenotyping EV from each contributing cell type here cartilage degradation assays could also be performed using EV from synovial fluid and from other suspected cellular sources. The linking to function may aid in stratifying and identifying EV origin. I have learnt valuable lessons on the pitfalls of identifying EV signatures from cells of similar parental origin, my new work builds on EV stratification in another complex biofluid where the contributing parental cells are more diverse and I am now investigating whether ExoView can aid in identifying EV of different cellular origins.

In my current post-doctoral position, I still find myself measuring EV and I still find the ExoView to be the single most data-rich measurement method, no other instrument I have yet tried can measure EV without purification. I am currently involved in a cross-

centre study investigating multiple EV measurement technologies (ExoView, NanoFCM, NTA, high resolution microscopy, flow cytometry, DLS, ImageStream), to try and establish how EV measurement technologies compare to each other. For that study I will be using a GFP tagged biological EV standard and hope to assess the similarity and differences in measurement outputs. Separately I am leading an investigation into synovial fluid EV measurement comparisons across similar measurement platforms with the addition of a fairly new instrument that claims to be able to measure EV in plasma- WhiteFoxTM. Due to the long period of time where ExoView has been unique in it's power to measure EV directly there have been limited opportunities to perform comparative studies and this new study aims to compare isolated EV across ExoView and other platforms but also EV in their native state where possible.

Overall, I think ExoView is an excellent technology that can be used to measure single EV within biofluids without any purification requirement. The use of a capture-system targeting EV-enriched markers allows for broad but not complete EV capture. ExoView combined with custom capture antibodies can be used to research rare or specific EV subpopulations in health and disease. However, it is not the candidate gold-standard EV measurement platform. The ideal gold-standard measurement platform will have the ability to specifically enumerate all EV in complex biofluids without any bias introduced by selection of EV of specific phenotypes. Ideally, this future instrument would be non-destructive and allow for EV recovery, where they could be measured or used for other purposes. It needs to be high throughput, operator independent and allow for the investigation of EV and their cargo at single-EV resolution. Although

ExoView can measure protein EV cargo, it is limited in its ability to measure RNA cargo [408]. I believe that next-generation microfluidic devices may be able to encapsulate what ExoView does well and bridge the gap towards gold standard EV measurement. Alternatively, future NanoFCM models may hold promise, the current models are limited to two colour fluorescence and can only be triggered on side scatter. It is not clear why fluorescent triggering is not capable with NanoFCM, but fluorescent triggering and upwards of five colour fluorescence is where NanoFCM needs to evolve to be getting close to the gold-standard EV method. NanoFCM could foreseeably be combined with technology similar to cell sorting, but at the level of single EV. NanoFCM with EV sorting capability would yield massive improvements to EV research. In the realm of the clinic, EV measurement needs robust and specific markers that are disease specific, if these can be found and easily detected I can envision bench-top devices in the future that are as routine in use as full blood counters.

6.2 Conclusion

This thesis shows the potential of ExoView in capturing and measuring EV in plasma without the need for purification. Whilst this is a leap forward in EV measurement, ExoView is biased in the EV populations that are captured although can be customized to capture specific subpopulations. ExoView is likely to be a staple in the EV biologist's measurement arsenal for some time. Future methodologies must prioritise the capacity to be both specific for rare EV and broad enough to capture and phenotype all EV.

Chapter 7. References

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